PCT / IN03 / 00204

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THE PATENTS ACT, 1970

IT IS HEREBY CERTIFIED THAT, the annex is a true copy of Application & Complete Specification filed on 31/05/2002 in respect of Patent Application No. 487/MUM/2002 of Secretary Department of Atomic Energy, Govt. of India, Anushkthi Bhavan, Chatrapathi Shivaji Maharaj Marg, Mumbai : 400 001, Maharashtra, India.

This certificate is issued under the powers vested in me under Section 147 (1) of the Patents Act, 1970.

Dated this 16th day of July 2003

(N. K. GARG)
ASST. CONTROLLER OF PATENTS & DESIGNS

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FORM 1
THE PATENTS ACT, 1970
APPLICATION FOR GRANT OF A PATENT (Section5(2),7 and Rule 33A)

(IN TRIPLICATE)

Secretary,
We/Department of Atomic Energy, Govt of India, Anushkthi Bhavan, Chatrapathi Shvaji Maharaj Marg, Mumbai 400001, Maharashtra, India hereby declare

1. (a) that we are in possession of an invention titled

" An improved method of detection of target nucleic acid sequence by nucleic acid amplification"

(b) that the **complete specification** relating to this invention is filed with this application.

(c) that there is no lawful ground of objection to the grant of a patent to us

2. further declare that the inventors for the said invention are **Shri Amirul Islam, Scientific Officer and Smt. Papia Hazra, Scientific Officer all of JONAKI, Board of Radiation and Isotope Technology(BRIT), Dept. of Atomic Energy, CCMB Campus, Hyderabad- 500 007 all Indian citizens**

3. We claim the priority from the application(s) filed in convention countries, particulars of which are as follows : **NONE.**

4. We state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which we are the applicant/patentee : **NOT APPLICABLE.**

5. We state that the application is divided out of our application, the particulars of which are given below and pray that this application deemed to have been filed on _____ under section 16 of the act : **NOT APPLICABLE.**

6. that we are the assignee of the true and first inventors

7. that our address for service in India is as follows ; **C/o Mr. Majumdar, M/s S. Majumdar & Co Patent & Trade Marks Attorneys, 5, Harish Mukherje Road, Calcutta-700025.**

487/mum/2002
31/5/2002

8. We, the true and first inventors for this invention declare that the applicant herein is my assignee

Shri Amirul Islam, Scientific Officer, JONAKI, Board of Radiation and Isotope Technology(BRIT), Dept . of Atomic Energy, CCMB Campus, Hyderabad- 500 007, Indian citizen

(Signed) Amirul Islam

Smt. Papia Hazra, Scientific Officer , JONAKI, Board of Radiation and Isotope Technology(BRIT), Dept . of Atomic Energy, CCMB Campus, Hyderabad- 500 007, Indian citizen

(Signed) Papia Hazra

9. that to the best of our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

10. following are the attachments with the application

(a) complete specification (in triplicate)
(b) abstract of the invention (in triplicate)
(c) drawings, (~~in triplicate~~) (38 sheets)
(d) Statement of Uundertaking (Form 3)
(e) Power of Attorney
(f) fee Rs. 5000.00 (five thousand rupees only) in bank draft bearing no. 787493 dated 28/05/02 drawn on Standard Chartered Bank

We request that a patent may be granted to us for the said invention

Dated this 24th day of May 2002.

Dinesh

(Signed) दिनेश भाटिया / Dinesh Bhatia
उप सचिव (ई आर) / Deputy Secretary (ER)
भारत सरकार / Government of India
परमाणु ऊर्जा विभाग / Department of Atomic Energy
छ.शि म. मार्ग / C.S.M. Marg
मुंबई - 400 001 / Mumbai - 400 001.

The Controller of patents
The patents Office
Mumbai.

ORIGINAL

FORM - 2

THE PATENTS ACT, 1970

(39 OF 1970)

COMPLETE SPECIFICATION

(See Section 10)

1. TITLE OF INVENTION

AN IMPROVED METHOD OF DETECTION OF TARGET NUCLEIC ACID SEQUENCE BY NUCLEIC ACID AMPLIFICATION

2. Secretary, DEPARTMENT OF ATOMIC ENERGY, Government of India, Anushakti Bhavan, Chhatrapati Shivaji Maharaj Marg, Mumbai - 400 001; Maharashtra, India.

The following specification particularly describes the nature of the invention and the manner in which it is to be performed.

Field of invention

The present invention relates to a method of detection of a target nucleic acid sequence by nucleic acid amplification reaction and to a kit used for such detection of target nucleic acid sequence. It would be possible by way of the above method to detect and quantify polynucleotide sequences in a sample of biological and non-biological material by way of a very sensitive, rapid and reliable method with improved specificity and reliability for the detection of polynucleotide sequence.

Back Ground of the Invention

The analysis and detection of minute quantities of substances in biological and non-biological samples has become a routine practice in chemical diagnostic and analytical laboratories. These detection techniques can be divided into two major classes. (1) Those based on ligand-receptor interaction (e.g. immunoassay – based techniques), and (2) Those based on nucleic acid hybridization (polynucleotides or oligonucleotide sequence – based techniques).

Immunoassay- based techniques involve a sequence of steps based on non-covalent binding of an antibody and antigen complementary to it. In these techniques analytes of concentration as low as a nanomole can be detected.

The present trend is towards the detection of polynucleotide sequences for analyte analysis. Polynucleotide sequence based detection of analytes requires detection limit as low as attomole. Polynucleotide sequence based techniques are mostly based on hybridization, the non-covalent binding in accordance with Watson-Crick base pairing of a labeled polynucleotide sequence to a complementary sequence of the analyte. Such polynucleotide sequence based detection techniques are divided into two categories: (1) Heterogeneous phase detection, (in which the analyte is fixed to a solid phase support such as nylon, cellulose etc., the labeled oligonucleotide is hybridized to the analyte, are washed in a number of steps and finally detected by colorimetric / color precipitation/Chemiluminescence/ bioluminescence/fluorescence/ ELISA), and 22(2) Homogeneous phase detection, in which detection is carried out in solution.

Heterogeneous phase detection techniques normally give higher sensitivity, i.e., detection of lower quantity of the analyte in comparison to homogeneous phase detection. But heterogeneous phase reaction is slow and more over involve many washings and other separation steps before final detection; hence those are more time consuming and complex. On the other hand, homogeneous phase detections are very simple, fast, easy to automate, easy to handle and adapt in any laboratory. Only disadvantage is its lower sensitivity. The detections are mostly fluorescence spectrophotometry based. With the advent of polymerase chain reaction, RT - PCR and ligase chain reactions, which are again homogeneous phase techniques, the target polynucleotide/oligonucleotide sequence can be amplified $10^6 - 10^8$ times, thus even a less sensitive detection method coupled with a target polynucleotide sequence amplification method will give a very high sensitivity. Hence, a homogenous phase detection method in conjunction with any of the above nucleic acid amplification techniques is ideal for detection and quantification of polynucleotide / oligonucleotide sequences in analyte. Molecular energy transfer and particularly, fluorescence resonance energy transfer (FRET), based detection methods are ideal for homogenous phase detection. This method enables, fast detection and quantitation without any separation step. Though this detection method alone is less sensitive than many other sensitive detections techniques, when used in conjunction with any of the above polynucleotide amplification techniques give very high sensitivity equal to or greater than the sensitivities of other techniques and hence, achieves the sensitivity requirement of polynucleotide sequence detection and quantitation in analyte and ultimately giving sensitivity higher than that of heterogeneous phase detection methods.

By this method even a few copies of the polynucleotide sequence can be detected which is not possible by other methods. The method has all the advantages of homogenous phase (solution phase) detection methods. It is again a non-radioactive method, so no health hazards, no radioactive waste management problem and can be adapted in any laboratory. Moreover, the method is suitable for one tube detection without opening or further manipulation and capable of both qualitative and quantitative detection.

FRET labels were first introduced in 1970's in immunofluorescence assay for detection of specific antigen (Ulman et al J.Biochem (1970), 251, 4172-4178, U.S. patent Nos.2, 998,943; 3996,345; 4160,016; 4174,384; and 4,199,559). Later in the 1980's many methods of detecting DNA and RNA by homogenous sequence specific hybridization using energy transfer and fluorescence quenching labels were developed (Heller et al U.S. patent Nos. 4,996,143; 5,532,129; and 5,565,322; European patent No. 070,685; year 1983 and others). In European patent 070, 685, year 1983 " Light emitting polynucleotide hybridization diagnostic method", Heller *et. al.* described the detection of a longer single stranded DNA target using two oligonucleotide probes, one labeled with a donor fluorophore at 5' end and the other labeled with an acceptor at its 3' end such that on hybridization two labels are placed close resulting in fluorescence energy transfer.

In European patent 229, 943, year 1987, "Fluorescent strokes shift probes for polynucleotide hybridization assay", Heller et al described the same scheme with specified distances between the donor and acceptor for maximum FRET. They also disclosed that the donor and acceptor could be located on the same probe.

A later demonstration used oligonucleotides with terminal labels (5'and 3') of fluorescein and tetramethyl, rhodamine (Cardullo *et.al.* 1988, Detection of nucleic acid hybridization by non-radioactive fluorescence resonance energy transfer, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8790-8794). Fluorescence of fluorescein label was reduced 71% on addition of the target DNA. In a recent variation on this format, a strong metal chelator (fluorescent acceptor) was attached to the 5'-terminus of one oligonucleotide and a weaker fluorescent chelator (donor) was attached to the 3'- terminus of the second oligonucleotide (Oser and Valet, 1990, Non-radioactive assay of DNA hybridization by DNA template mediated formation of a ternary Tb (III) complex in pure liquid phase, *Angew Chem. Int. Ed.Engl.* **29**,1167-69).

In U.S. patent application no 661,071; year 1993 and PCT application PCT/US 92/1592 publication no WO 92/14845 entitled, "Diagnosing cystic fibrosis and other genetic diseases using fluorescence resonance energy transfer" a DNA

hybridization based detection system similar to that of Heller et. Al (European patent 070,685; year 1983) was disclosed.

In a second assay format referred to as a competitive assay one probe is labeled on its 3' terminus, and the other probe is labeled on its 5' terminus and they hybridize to each other resulting in fluorescence quenching (European patent 232,967; year 1987, Morrison *et. al.* Solution phase detection of polynucleotides using interacting fluorescent labels and competitive hybridization, *Anal. Biochem.* **183**, 231-244). In target detection there is competition between the probes and the target. More the target strands present, more the probe strands hybridize to the target strands and lesser the number of donor and acceptor placed next to one another by probe to probe hybridization. The presence of target DNA is detected as increased emission from donor due to reduced quenching, and reduced emission from acceptor due to reduced energy transfer.

In "Rapid detection and identification of infections agents" pages 245 – 256 in Chemiluminescent and fluorescent probes for DNA hybridization systems ed. by D.T. Kingsbury and S.Falkow, Academic press; Heller and Morrison described another nucleic acid detection format in which one fluorophore (F) labeled single stranded probe and a dye (Q) that preferentially binds to double stranded DNA is used. In presence of the target DNA, the probe hybridizes to the target and acceptor (Q) binds to the resulting double helical region, thereby placing Q near F and allowing fluorescence energy transfer or fluorescence quenching.

FRET has also been used for studying the hybridization process (Morrison and Stols 1989, The application of fluorophore labeled DNA to the study of hybridization kinetics and thermodynamics, *Biophys. Jl,* **55**, 419; A sensitive fluorescence based thermodynamic and kinetic measurement of DNA hybridization in solution, *Biochem.* **32**, 3095-3104, Perkins et.al.,1993, Accelerated displacement of duplex DNA strands by a synthetic circular oligodeoxynucleotide, *J. Che. Soc. Chem. Comm.* 215-216).

Distance relationship in double helical structures has also been measured using energy transfer between two fluorescent labels attached to DNA oligomers

(Cardullo *et.al.* 1988, *Proc. Natl. Acad. Sci. USA,* **85**, 8790-8794; Cooper and Haserman, 1990, Analysis of fluorescence energy transfer in duplex and branched DNA molecules, *Biochem.* **29**, 9261-9268, Ozaki and Mc. Laughlin, 1992, The estimation of distance between specific backbone-labeled sites in DNA using fluorescence resonance energy transfer, *Nucl. Acids Res*, **20**, 5205-5214; Clegg *et. al.* Observing the helical geometry of double stranded DNA in solution by fluorescence resonance energy transfer *Proc. Natl Acad. Sci. U.S.A,* **90**, 2994-2998).

Detection and quantitation of nucleic acids by polymerase chain reaction (PCR) and molecular/fluorescence resonance energy transfer: -

For clinical and other analyses, detection requirement below, one attomole of polynucleotides are common. Fortunately, there are polynucleotide amplification systems available that amplify the target polynucleotides. Among those are polymerase chain reaction (PCR), reverse transcription coupled polymerase chain reaction (RT PCR), ligase chain reaction (LCR), Nucleic acid sequence based amplification (NASBA), Triamplification, Strand displacement amplification (SDA) and others. The PCR (Mullis and Faloona, 1987, Methods in Enzymology 155, 335-350) is the best known and most studied of the amplification systems. All the above nucleic acid amplification methods are capable of producing well over a million copies of the target polynucleotide originally present in the sample.

All the above nucleic acid amplification methods are homogeneous phase polynucleotide target amplification techniques. The amplified product should be detected for the complete polynucleotide analyte detection The detection technique should be based on homogeneous phase detection and the detection method need not be very sensitive because the target has already been amplified to a high level. Thus coupling of any of the above nucleic acid amplification of the target polynucleotide with a homogeneous phase detection of the amplified product allows for a completely homogeneous assay having the advantages of both high sensitivity and simplicity.

Commonly nucleic acid amplification product detection requires the separation of the product from the unreacted primers and nucleotides. Agarose gel

electrophoresis is the most commonly used technique for this and is based on size differentiation. The detection is by ethidium bromide staining of the gel. Alternatively, the amplification product is immobilized on a solid surface and detected with a labeled product. The unreacted primers, probes and nucleotides are washed away. One of the problems associated with the detection of the amplified product by the above two methods is carry over contamination of the amplified product. Since there is high level of target sequence amplification, while opening the tubes containing the amplified product, the amplified product gets released in the aerosol form and contaminates the laboratory. Subsequent amplification reactions will have the contamination of this target sequence (because of high amplification), thus giving false positive results. However, MET / FRET allows detection of amplification product without separation of the unutilized primers, probes and nucleotides. Hence there is no need to open the amplification reaction tube and no carryover contamination problem. Moreover, MET / FRET is solution phase homogeneous detection technique, hence very simple, fast and efficient detection method, and amenable to automation. Prior to the present invention, five methods for monitoring the amplification product using MET / FRET has been described.

The first method reported for the detection of amplification product without prior separation is based on the 5'-exonuclease degradation of doubly labeled probe during PCR amplification, referred to as the Taq Man assay (Holland *et.al.,* 1991, *Proc. Natl. Acad. Sci U.S.A.* **88,** 7276-7280; Lee *et. al.,* 1993, *Nucl. Acids. Res,* **21**, 3761-3766. In this assay during the annealing step of the amplification reaction the doubly labeled fluorogenic probe hybridizes to the complementary target sequence. The 5'-exonuclease activity of the enzyme Taq DNA polymerase used for amplification degrades the hybridized probe. The probe is degraded only when it hybridizes to the target sequence being amplified. One of the labels is a fluorescent donor and the other is a quencher. In the labeled probe fluorescence of the donor is quenched. On degradation of the probe quenching is removed and the donor fluorophore fluoresce resulting in the detection of the target. In Taq man assay the donor and the quencher are located preferably at the two ends of the probe, i.e., the 5' and 3' ends; because the 5' to 3' exonuclease hydrolysis of the probe can be achieved only when these

two labels are not too close to each other (Lyamichev et.al 1993, *Science*, **260**, 778-783). This is a serious drawback of this assay method. The efficiency of energy transfer between the donor and the quencher (acceptor) decreases with increase of distance between two by inverse sixth power of the distance. Since, the quencher cannot be placed close to the donor, most efficient quenching of the donor (reporter) cannot be achieved. As a result, background fluorescence from unhybridized probes will be high.

Further in Taq man assay the amplification product is not measured directly rather an event related to the amplification, i.e., the hydrolysis of the probe that hybridizes to the amplification product between the two primer sequences. Following problems are associated with this method as discussed in the U.S. patent 5,866,336. First, hybridization will never be quantitative unless the labeled oligonucleotide probe is in great excess. This in turn will result in high background (because quenching is never quantitative and moreover Taq man probes are not quenched efficiently). Secondly, oligonucleotide probes hybridized to the middle of the target DNA will slow down the PCR amplification process. Thirdly, all of the oligonucleotide probes hybridized to the amplified product will not be subjected to 5'-3' exonuclease hydrolysis; some will be displaced without hydrolysis resulting in loss of signal. Fourthly, probes non-specifically hybridized to the portion of the amplified product other than the targeted region will give fluorescence signal resulting in over estimate of the analyte. This method is one tube method, hence does not have carryover contamination problem.

Another method of detection of amplification product using FRET is the molecular beacon probe method described by Tyagi and Kramer, 1996, *Nature Biotech.***14**, 303-309, Lizardi *et.al.* U.S. patents 5,119,801 and 5,312,728). This method is again based on oligonucleotide probe hybridization. The oligonucleotide probes (molecular beacons) are of hair - pin (loop and stem) configuration. On one end of the oligonucleotide probe (either 5' or 3- end) there is a donor fluorophore, and on the other end an acceptor moiety, which is a quencher.

The molecular beacon probes are in strained conformation. Whenever, the loop portion contact perfectly matched target sequence it forms a stable hybrid

destabilizing the stem structure, resulting in an open conformation of the probe separating the donor fluorophore from the acceptor (quencher). Otherwise, in the absence of the target sequence, the beacon probe is in its closed conformation (hair-pin), in which the fluorescence of the donor fluorophore remains quenched.

When employed in PCR assay, the molecular beacons, which hybridize to one strand of PCR products are in open conformation and emit detectable fluorescence. Those molecular beacons that remain unhybridized will not fluoresce. The amount of fluorescence will increase as more and more PCR products are formed, giving a measure of the progress of PCR and ultimately measure of the analyte in the sample.

Since, this method is solely based on probe hybridization like Taq man assay it also has the drawbacks of hybridization methods. Though high specificity and sensitivity is claimed, there still remains certain amount of non-specificity. It is unlikely that the beacon probe will quantitatively hybridize to the particular strand and particular site only for which it is designed, particularly when the PCR product is much longer than the beacon. It can also hybridize to other non-template nucleic acid sequences present in the sample and to non-specifically amplified products.

Even those probes that are hybridized to the template could be displaced by the second DNA strand under synthesis or polymerization during annealing or annealing cum extension step (two step amplification i.e., denaturation and extension required for some amplifications) over a short period of time; as a result this method cannot be quantitative.

Another major draw back of the method is that the measurement is based on removal of quenching of the donor fluorophore. Quenching can never be quantitative. As a result the fluorescence background will be high. Since the method is based on hybridization of the probe to the amplified product, quantitative hybridization of the probe will require higher concentration of probe that in turn will increase fluorescence background further (as discussed in US Patent No. 5,866,336; year 1999). In addition dissociation of the donor or the

quencher from the probe during PCR process due to the break down of the linkage between the fluorophore and / or the quencher will increase the nonspecific signal background resulting in low signal to background ratio and thus limiting detection limit (lower sensitivity of detection).

Non – specific products do form in PCR nucleic acid amplification reaction at some stage or other particularly with complex samples. Some non – specific products can get extended from its 3' end through the 3' end upto the 5' end of the labeled hair – pin beacon probe. This extension through the hair – pin probe would be in right orientation and can anneal with the target sequence and extend in the next cycle of the PCR amplification, to the respective end of the amplification product, the resulting product can be amplified exponentially thereafter. Thus a non – specific amplification product generated at some stage of amplification can result in exponentially amplified product to which hair – pin beacon probes can hybridize thus giving a higher estimate of the target sequence.

Still one more disadvantage with the method is that the method is based on the hybridization of the probe between the two primer sequences of the amplified product, which will hinder or slow down the amplification process. Moreover, for multiplexing, i.e., detection of multiple targets in single reaction tube will require multiple light sources and hence costlier instrument.

In 1995, Jingyere Ju et. al. PNAS 1995, **92**, 4347-4351, (U.S.patent No. 5707804, 1998) developed fluorescence energy transfer primers for DNA sequencing. In 1995, Wang et. al. *Anal chem.*1995, **67**, 1197-1203) used the fluorescence energy transfer primers in PCR detection of simple tandem repeat sequences. In both the methods fluorescent energy transfer (FRET) primers were incorporated into the PCR amplification product. However, their objective was to improve the fluorescence intensity of non – radioactive sequencing ladder and to detect different repeat lengths in individual samples respectively. Resolution of different repeat lengths requires separation and they used capillary electrophoresis for the same. Quantitation of the PCR product was not their goal, so quantitation based on FRET primer incorporation into PCR product was not

reported. Wang et al in their U.S. patent bearing the No. 5,348,853 described a fluorescence energy transfer based method for the detection and quantitation of nucleic acid target using PCR amplification. In this method an asymmetric PCR amplification of a target sequence was carried out using one amplification primer in excess such that one of the target strands is significantly overproduced. A primer duplex labeled with a donor and an acceptor fluorophores, complementary to the over produced target was used to prime a semi-nested reaction in concert with the excess primer. As the semi nested amplification proceed the labeled reverse primer start dissociating from the primer duplex and getting incorporated into the amplified product. The incorporation of the labeled reverse primer into the amplification product was measured by the disruption of the energy transfer between the donor and the acceptor of the primer duplexes and through the decrease in the fluorescence intensity of the acceptor. The decrease in the fluorescence intensity was proportional to the initial target dosage and the extent of amplification. This method is actually a modification of the method of European patent 232,967 year 1987, Anal. Biochem. 183, 231 – 244.

However, in this method the amplified product was detected by incorporation of a labeled reverse primer into the amplification product, which again was detected indirectly through the dissociation of the labeled reverse primer from the FRET labeled primer duplex rather than directly detecting the labeled amplification product through increase in donor fluorophore emission. This method depended on decrease in emission from the acceptor fluorophore rather than the increase in donor fluorophore emission. Hence the signal to noise ratio was low. Moreover the method used a preliminary amplification step (asymmetric PCR) to increase the initial target concentration and subsequent addition of labeled primer duplex which complicates the process as well as involves opening of the tube.

Another method based on FRET has been described by Nazarenko et.al. in their U.S. patent no.5866,336 published in year 1999 and US Patent No. 6,117,635 in year 2000 . The method is based on the incorporation of FRET primer /primers into the PCR amplification product. One of the two amplification primers is a FRET primer, i.e. labeled with a fluorescent donor moiety and an acceptor moiety; the acceptor moiety can be another fluorophore or quencher. The

detection and quantitation is based on the incorporation of the fluorescently labeled primer into the PCR amplification product. The FRET primer either does not give fluorescence or fluoresce at different wavelength when it is not incorporated into an amplification product. Here PCR amplification product is measured directly by measuring the amount of fluorescence emitted by those products into which the fluorescently labeled primer has been incorporated.

In preferred embodiment hair - pin primers are used and the measurements are made by measuring increase or decrease of fluorescence and this measurement can be carried out in real time, i.e. as the PCR amplification progresses with time. On the other hand when linear primer/primers labeled with a donor fluorophore and an acceptor fluorophore, the fluorescence from the primers incorporated into the amplification products is measured after degrading unutilized primers with a 5'→3' exonuclease activity at the end of the amplification. In this embodiment end point rather than real time measurement is carried out and the measurement is through decrease in fluorescence. Similarly, in case of triamplification using labeled oligonucleotides, the amplified products into which the labeled blocking primer and the labeled amplification primer have been incorporated is detected after degradation of the unutilized oligonucleotides by 5'→3' exonuclease treatment or after separation (denaturation) of the labeled oligonucleotides by heating at higher temp. (75^0C).

This method is a modification of the method of Wang et. al. US Patent No. 5,348,853 which is based on the incorporation of a fluorophore labeled reverse primer into amplification product and measurement of the same by an indirect method rather than a direct method. In this method the authors have measured the amplification product directly by measuring the increase in the fluorescence emission of the donor fluorphore. This method also has many drawbacks. First the preferred detection primer is a hair - pin quenched primer. Fluorescence signals are generated by removal of quenching as a result of incorporation of the primer into the amplification product. Quenching (static and dynamic) can never be quantitative and will result in high background as discussed by the authors Nazarenko et al in their U.S. patent 5860,336 and Morrison L.E in the chapter; Detection of energy transfer and fluorescence quenching in the book, Nonisotopic

probing, blotting and sequencing, 1995, pages 442, 444, 445, 490 edited by Kricka L J and published by Academic Press.

In case of triamplification a blocking primer and one of the amplification primers complementary to the blocking primer are labeled separately with a donor and an acceptor in order to give FRET when incorporated into the amplification product; the acceptor being a fluorophore. The acceptor is a fluorophore and sensitized emission is measured for detection and / or quantitation. Problem in using an acceptor fluorophore in FRET is that the acceptor fluorophore gets excited to sizeable extent by the light used for exciting the donor thus resulting in considerable background. This is a major problem in measurement based on sensitized emission of an acceptor moiety. Hence both in case of 5'→3' exonuclease degradation and heating at high temperature used for detection of the amplification product there will be sizeable background because of this excitation of the acceptor by the donor excitation light. The same problem will also be there incase where linear amplification primers doubly labeled with a donor fluorophore and an acceptor fluorophore is used, even after 5'→3' exonuclease degradation of the unutilized primers, (which is in much excess) the acceptor fluorophore released from the primer will be partially excited by the light used for excitation of the donor fluorophore, thus resulting in sizeable background (Morrison, L. E. In the chapter "Detection of energy transfer and fluorescence quenching" in the book, Nonisotopic probing, blotting and sequencing To 1995, pages 442, 444, 445, 490 edited by Kricka L J and published by Academic Press.

Second, the preferred hair - pin primers used have long non-target specific sequence (from universal hair-pin configuration) at the 5' end of the target specific primer (Primer-1). Designing good specific primers, which do not find complementarity in parts of the complex genomic material other than the region designed for, is a major hurdle in PCR amplification and detection of nucleic acids targets. Addition of non-target specific sequence at 5' end of a specific primer brings non-specificity due to the fact that at the annealing temperature of the target specific primer, the primer with added sequence may anneal in many other places on the complex genomic materials of the sample (which may contain a mixture of one or more contaminating genomic material in addition to the

genomic material of the target sequence) resulting in non-specific product; while increase in annealing temperature to avoid non-specific product may result in failure of the amplification reaction.

Designing a good suitable amplification primer with long non-target specific sequence at the 5'-end of target specific primer sequence not giving non – specific product for amplifying a target from a complex sample, such as genomic DNA, total RNA from bacteria, fungi, plants and animals is difficult or limited. And taking into account the possibility of contamination from many of these materials in the sample, job is much more difficult. This is a limitation of the method. There may be a success in case of a specific target in a specific sample but in general there will be problem in the amplification. Moreover, in view of the practical problem of temperature variation from well to well in the same thermal cycler, between thermal cycler to thermal cycler and laboratory to laboratory, amplifying a specific target polynucleotide sequence with the above type of primers free of any non – specific amplification product would be difficult and limited. Again in this method the quantitation is based on incorporation of the above-labeled primer into the amplification product. Hence there is bound to be over estimate due to non-specificity. Also any primer extension specific or non-specific resulting either in amplification product or abortive amplification will result in signal generation thus giving an over estimate of the target. Avoiding non – specific amplification product in PCR amplification is real problem. On the other hand use of higher annealing temperature to increase specificity of amplification to overcome above problem will result in amplification failure and hence lowering of sensitivity of detection of the target.

The amplification in PCR is tremendous 10^6 times in 20 cycles. That is why PCR amplification based detection is a highly sensitive method. High stringency annealing of the primer increases specificity. Again, higher the specificity, higher is the PCR amplification failure, hence lesser overall sensitivity of the method. Addition of the thermostable DNA polymerase enzyme in the PCR little more than the required give a lot of non-specific product and it is difficult to add always exactly the required quantity of the enzyme since the enzyme comes in 50% glycerol solution, which is a viscous solution and it is difficult to add exact volume

of viscous solution. Here, is also the scope for personal error. It is also observed that different preparations of thermostable DNA polymerases from different sources give different amounts of non – specific amplification products. The present method is solely, dependent on the incorporation of labeled primer into the amplification product; taking this and non-specificity of amplification particularly with the said hair - pin primer, into account the method will give an over estimate of the target.

The non-specificity problem will be less with properly designed triamplification and linear primer incorporation but there are background problem, additional manipulations such as ligation of triamplification oligonucleotide, heating at higher temp to destabilize the hybridization between the labeled amplification primer and the labeled triamplification oligonucleotide, 5'→3' exonuclease digestion of free oligonucleotide in case of triamplification and 5'→3' exonuclease digestion of the unutilized labeled linear primers, which are additional steps, making the method complex, error and failure prone. More over, these measurements can be carried out at the end point only; no real time measurement is possible and hence no reliable quantitation. The authors have also mentioned about FRET in triamplification, and in linear FRET primers used in amplification in conjunction with 5'→3' exonuclease digestion and heating at higher temperature for the detection and quantitation of polynucleotide target but use of FRET between donor and acceptor moieties on two amplification primers have not been put forward.

Authors have pointed out background problem associated with the use of higher concentration of labeled molecular beacon probes (Tyagi and Kramer; 1996, Nature Biotech, 14, 303 – 309; Lizardi et al U S Patents 5,119,801 and 5,312,728) which are normally used at a concentration of 0.5 □M. The same background problem will be there in the authors' method with the use of labeled amplification primers, which are normally used at a concentration of 0.5 □M (range 0.1-1 □M). Only 1 to 20 percent of the primers normally get incorporated into the PCR amplification product. Such a large excess of primers will give high background because fluorescence quenching in the primers cannot be quantitative. During the PCR amplification a certain percentage of the fluorophore

/ quencher or donor / acceptor whatever may be the case will get detached from the labeled amplification primer due to the breakdown of the linkage between the fluorophore and / or the quencher or the donor / acceptor as the case may be, thus resulting in background, inaccuracy in quantitation and rise in detection limit i.e., lower sensitivity. Moreover in preferred embodiment multiplexing, i.e., detection of multiple targets in single reaction tube will require multiple light source and hence costlier instrument.

Primer dimer formation by the hair – pin labeled primer of the preferred embodiment with itself (homodimer) and the second primer (heterodimer) can generate signal resulting in background. Moreover, any non – specific product formed during any stage particularly at the beginning of amplification can get extended through the labeled hair – pin primer, the way primer dimers are formed, resulting in product that amplifies exponentially in the remaining cycles thus resulting in high background and low signal to noise ratio. This may be a major reason for the low signal to noise ratio (35) achieved in this method as disclosed in US Patent Nos.5866336 and 6117635 in comparison to expected signal to noise ratio of around 200.

Recently, an US Patent No. 6,174,670 has been published where FRET has been used for the detection or quantitation of nucleic acid target sequence. In this method authors Wittwer et. al. have used two hybridization probes one labeled at its 3' end and the other labeled at its 5' end separately with a donor or an acceptor FRET moiety so that when these two probes hybridize to the strand of the amplification product against which these two probes are configured the donor and the acceptor moieties on two probes come in proximity such that FRET can take place between the two moieties and measurement of the increase in acceptor emission or decrease in donor emission gives the measurement of the amplification product or the progress of the amplification process. The authors have also mentioned the use of one of the amplification primers as one of the above two hybridization probes. This method is an extension of the method 'Non – amplified nucleic acid target sequence detection' of European patent 070,685 year 1983, US patents 4,996,143; 5,532,129; 5,565,322 to the PCR amplified nucleic acid target sequence detection. This method also has a lot of

drawbacks. In this method two probes configured for hybridization to the amplification product will hamper the amplification reaction resulting in sluggish PCR amplification reaction. The probe / probes can easily be displaced by the polymerase before the FRET signal can be measured thus resulting in a lower estimate of the amplification product or the amplification reaction. In this method there will be a lot of background because of the excitation of the acceptor FRET moiety by the light used for exciting the donor fluorophore. Because of this background the method can be used for a very limited number of donor acceptor pair, that is why the authors have claimed for only Fluorescein as donor and Cy – 5 or Cy – 5.5 as acceptor FRET pair.

It is clear that all of the prior art methods for detecting amplification products are having more than one shortcoming. None of the prior art methods are suitable for trouble free reliable detection or quantitation of the nucleic acid amplification product. So there is a need for improvement. PCR based detection methods are simple, rapid and highly sensitive. Any PCR based assay should be highly specific and at the same time highly reliable. Because of high level of amplification of nucleic acid there is associated carryover contamination problem. One way of avoiding this is not to open the reaction tube for detection, i.e. to adapt a close tube detection format, which can be achieved by using FRET, based detection.

Any detection or quantitation method may have success in certain cases but for it to be adapted universally in many laboratories need to have proper control. For example, the Random Amplification Polymorphic DNA (RAPD) technique can be cited, which had met initial success but due to lack of proper control is not used much these days. None of the above methods whether it is molecular beacon method or labeled primer incorporation method meet this requirement. Hence there is need for the development of a FRET based close-tube format, simple direct low back ground, highly specific, highly sensitive quantitative and reliable method with proper control independent of personal error and sample type for the detection of the PCR amplification product.

Objects of invention

It is thus the basic object of the present invention to provide for an improved method for the detection and quantitation of polynucleotide sequences in sample of biological and non-biological material through target polynucleotide sequence amplification involving polymerase chain reaction which would be very sensitive, rapid and reliable method and bring about better sensitivity, specificity and reliability in the detection of polynucleotide sequences.

Another object of the present invention is to provide for improvement for detection and quantitation of polynucleotide sequences which would substantially reduce the possibility of amplification product carry over contamination and would be adaptable for wide range of nucleic acid amplification and real time measurement for the detection and quantitation of polynucleotide sequences in sample of biological and non\-biological material through target polynucleotide sequence amplification which would be highly reliable and take care of false positive result experienced in known art and enable carrying out of amplification detection with less PCR failure and less false negative result i.e. higher sensitivity of sample detection.

Yet further object of the present invention is to provide for the detection and quantitation of polynucleotide sequences in sample of biological and non\-biological material through target polynucleotide sequence amplification involving a closed tube format whereby the measurement can be carried out in real time both in homogeneous solution phase assay and semi-homogeneous/heterogeneous phase assay.

Yet further object of the present invention is to provide for a method of detection and quantitation of polynucleotide sequences in sample of biological and non-biological material through target polynucleotide sequence amplification which can be carried out on polynucleotides that may be present in any biological or non-biological sample, such as clinical samples, for example blood, urine, sputum, saliva, faeces, pus, semen, serum, other tissue samples, culture media,

fermentation broth and the like with or without pre-extraction of purification of analytes by known methods to concentrate nucleic acids.

Yet further object of the present invention is to provide for a method of detection and quantitation of polynucleotide sequences in sample of biological and non-biological material through target polynucleotide sequence amplification wherein use of amplification primers for amplification of an amplification product of the size close to that of primer dimer help in improving signal to noise ratio.

Yet further object of the present invention is to provide for a method of detection and quantitation of polynucleotide sequences in sample of biological and non-biological material through target polynucleotide sequence amplification whereby it would be possible to achieve a unique low background, improved higher signal to noise ratio generating signal measurement protocol which make the method of highest sensitivity in detection of nucleic acid sequences.

Yet another object of the invention is to provide a MET/FRET based one tube method for real time measurement or quantitation of target polynucleotide sequence.

Yet another object is to provide an improved method for detection and quantitation of polynucleotide sequence or sequences in a sample in very short time and in standard tube or microtitre plate format so that large number of sequences can be detected or quantitated in short time.

Yet another object is to detect the amplification product (of the size of primer dimmer or close to that) by utilizing intercalating fluorescent dyes like ethidium bromide, picogreen, SYBER TMGREEN 1,acridine orange, thiazole orange, chromomycin A3 and YO-PRO-1 and others

It is another object of the present invention to develop kits and labeled oligonucleotide amplification primer sets for the detection and/ or measurement of polynucleotide nucleic acid amplification products, polynucleotide nucleic acid target sequence in the sample which would favour effective and improved

detection and quantitation of polynucleotide sequences in samples of biological and non-biological materials.

Summary of the Invention

Thus according to one aspect of the present invention there is provided a method of detection of a target nucleic acid sequence by nucleic acid amplification reaction, comprising use of two oligonucleotides as a pair of primers for amplification of the said sequence, with one primer being labeled with a donor MET moiety, and the other primer being labeled with an acceptor MET moiety, the said donor and acceptor MET moieties belonging to a molecular energy transfer pair.

In the above-disclosed method of the invention the principle of molecular energy transfer (MET) between a donor moiety and an acceptor moiety is effectively utilized. In a preferred embodiment, the MET is fluorescence resonance energy transfer (FRET), in which the oligonucleotide primers are labeled with donor and acceptor moieties, the donor moiety is a fluorophore and the acceptor moiety is also a fluorophore. The fluorescence energy emitted by the donor moiety is absorbed by the acceptor moiety, which in turn releases the absorbed energy by emitting light at different wavelengths. The fluorescent emission from the acceptor gives the measure of the amplification reaction. Measurement of the reduction in donor fluorescence in addition to the acceptor fluorescence helps in counter checking the result. Further excitation of the acceptor, by acceptor specific excitation wavelength in case of use of hair – pin oligonucleotide, acceptor being part of it and measurement of its emission can give most accurate estimation of the amplification product or the target sequence present in the sample.

In accordance with the preferred aspect of the present invention the improvement of detection of target nucleic acid sequence by nucleic acid amplification comprises (i) use of two oligonucleotides as a pair of primers for amplification of said target sequence; (ii) the 3' ends of said pair of primers being separated from one another by 0-25 nucleotide pairs in the final amplification product; and (iii)

employment of time periods for denaturation, less than 10 seconds, annealing of less than 5 seconds and extension of 0 second in each cycle.

Another aspect of the present invention is to provide a kit for use in method of analogous detection and / or quantitation of target nucleic acid sequences present in the sample comprising

(a) a polymerase or polymerases

(b) a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence suitably labeled with a donor MET / FRET moiety at or near 3' end.

(c) a second oligonucleotide of sequence at 5' end of the first nucleotide sequence complementary to nucleotide sequence flanking the target nucleotide sequence or the segment of the target nucleotide sequence suitably labeled with an acceptor MET/FRET moiety at or near the 3' end

(d) deoxynucleotides in solution (water or buffer) or lyophilized.

(e) a reaction buffer for the nucleic acid reaction

wherein the first and second oligonucleotide sequences comprise the two primers (forward and reverse) of many nucleic acid amplification reactions and adapted to generate a detectable signal if the two oligonucleotides get incorporated into two opposite strands of amplified product and come in right proximity.

The above method is quantitative (less or no background), because measurement is based on molecular energy transfer and not based on fluorescence quenching used in prior art; and moreover non-specific signals due to non-specific incorporation of MET primer or primers into the non-specific amplification product is not measured in measuring process. The fluorescence / emission background due to direct excitation of the acceptor moiety by the light used for donor excitation is reduced by selecting acceptor whose excitation spectra overlap with emission spectra of the donor towards the longer wavelength end of the spectra or using quencher to quench acceptor emission in a hair-pin configuration or a configuration to serve the same purpose. The quencher can be radiative quencher or non – radiative quencher. The energy

transfer efficiency k_{et} is given by the expression $k_{et}=8.79x10^{-25}\ \kappa^2\ \phi_D\ K_D\ \eta^{-4}\ R^{-6}\ J$, where R is critical distance for 100% energy transfer, J is the overlap integral between the two spectra. Reduced J and reduced R will give same k_{et} value. Choosing an acceptor overlapping the donor spectra near the longer wavelength end and placed close to donor will give reasonably good energy transfer but with reduced excitation with light used for exciting the donor, i.e. reduced background. An acceptor with high quantum yield and high extinction coefficient of absorption is an acceptor of choice. Similarly a donor of high extinction coefficient of absorption and high quantum yield is a donor of choice.

In one embodiment both the oligonucleotide amplification primers, which are linear primers, are suitably labeled either with a donor moiety or an acceptor moiety at or near 3' ends preferably near 3' ends. The two primers are so labeled and designed that when the two primers get incorporated into the two opposite strands of the right amplification product the donor and the acceptor moiety are placed in correct proximity so as to result in MET between the two moieties. The donor is excited by its characteristic excitation light or radiation and the emission of the acceptor and optionally reduction in that of the donor is measured.

The two oligonucleotides are so designed that they bear a specific distance relationship between them so that they bring the donor and the acceptor moieties on them within the distance of 50% energy transfer between the donor and the acceptor when incorporated into the amplification product the donor and the acceptor MET moieties being in two opposite strands. This distance relationship gives the additional specificity of detection. The chances of these two labeled oligonucleotides coming within this distance is remote or nil. Moreover MET having inverse sixth power relationship with distance between the two moieties, the signal will drastically fall after the above 50% energy transfer distance. Hence specific amplification product only can give signal. If any non – specific amplification product is formed, that cannot give any signal

Another uniqueness of the method is that it utilizes the amplification of an amplification product of the size close to that of primer dimer i.e., the size of

forward primer plus the size of the reverse primer plus zero to twenty – five bases, for the detection and / or quantitation of a target sequence. Normally, in PCR amplification formation of primer dimers (homodimer and heterodimer) are avoided. It is speculated that if primer dimers are formed at any early stage of the amplification reaction the amplification of the primer dimer takes over the normal target amplification and the amplification of the primer dimer in PCR is very efficient. The present invention takes advantage of the highly efficient amplification of an amplification product of the size of the primer dimer. The amplification primers (forward and reverse) are designed for the amplification of a target segment of the size almost close of that of the primer dimer i.e., length of the forward primer plus the length of the reverse primer plus zero to twenty-five bases. Both the primers are tested for not forming primer dimers particularly heterodimers both by the standard softwares available as well as carrying out amplification reaction in absence of target sequence. These primers form a product when they contact a target sequence. Moreover, the method also utilizes another advantage of hair – pin primers, i.e., the hair – pin primers are highly efficient (a few times) over linear primers and gives specificity of primer annealing. Because of higher efficiency of hair – pin primers smaller amount, i.e., lower concentration of primers would be required that would in turn reduce the possibility of any primer dimer formation. Additionally the amplification of above size product being very efficient less amount of primer will be required for the amplification reaction. Moreover stable stem structures of hair – pin primers remain in closed configuration during annealing step in absence of target sequence thus preventing primer dimer formation further.

In a preferred embodiment the amplification primer labeled with the acceptor MET moiety at or near 3' end is in hair - pin configuration and a quencher which absorbs radiation or light covering the entire visible wavelength range or the emission of the acceptor is placed at or near the 5' end of the said primer so that the acceptor moiety and the quencher come very close to each other in the stem structure of the said hair - pin. The quencher on absorption of radiation / light may not give any further emission or may give emission at a wavelength different from that of the said acceptor moiety. The detection and / or quantitation are again based on the measurement of the emission from the acceptor MET moiety.

- Use of hair – pin primer with acceptor MET moiety label and quencher will largely reduce or eliminate the background due to excitation of the acceptor MET moiety by the radiation or light used to excite the donor MET moiety. Moreover, the hair – pin configuration of the primer will increase the specificity of primer annealing.

In another preferred embodiment both the donor and the acceptor moieties on the primers are quenched with a quencher like DABCYL, or other suitable quencher where the primers are in hair – pin configuration, labeled separately at or near their 3' end with the donor or the acceptor moiety. The quencher is attached at or near 5' ends of the oligonucleotides.

In another preferred embodiment both the donor and the acceptor moieties on the primers are quenched with a quencher like DABCYL, or other suitable quencher where the primers are labeled separately at or near their 3' ends preferably near their 3'ends, with the donor or the acceptor moiety and the MET moieties on the labeled oligonucleotide primers are kept quenched when not incorporated into the amplification product with the help of another two additional oligonucleotides suitably labeled with quencher at their 5' ends.

In another embodiment the method provides a lower background and higher increased signal to noise ratio and accurate quantitation of the amplification product or the target sequence and the use of the same in different methods of polynucleotide amplification including PCR, RT – PCR, NASBA, Ligase chain reaction, Strand displacement amplification (SDA), Triamplification.

In another embodiment the acceptor moiety on the primers are quenched with a quencher like DABCYL, which is the part of a second oligonucleotide partly / not complementary to the target sequence but fully complementary to the 3' end of the primer and attached to the 5'end of the primer through a short linker, which can be another oligonucleotide or an organic linker or linker and spacer at the 3' end (of the second oligonucleotide) so as to result in a stem structure bringing the acceptor moiety and the quencher very close resulting in quenching of the acceptor moiety. In an extension of this embodiment both the donor and the acceptor labeled primers can be provided quenched.

In still another embodiment two or four of the four oligonucleotide complementary to the two strands of target polynucleotides used in ligase chain reaction (LCR) are separately labeled with the donor and the acceptor MET pair moieties in such a configuration that MET can occur between partners only when the respective oligonucleotides are ligated by the enzyme polynucleotide ligase. In this embodiment of the invention the oligonucleotides are in hair-pin configuration with a fluorophore attached at or near one of the two ends and a non – radiative quencher DABCYL or the like at the other end so that the fluorescence of the unligated oligonucleotides remain quenched with the quencher like DABCYL and the donor is excited by the donor specific excitation light or radiation and the increase in acceptor emission is measured.

In a further embodiment one of the labeled amplification primers is fixed or attached at or near its 5' end to a solid support through a linker and a spacer while the other primer/primers are in solution phase in contact with the solid support, so that multiple/ large number of polynucleotide targets in the analyte can be analyzed simultaneously. Preferred solid supports are glass, silicon wafers, polystyrene, polypropylene but not excluding others and the preferred supports are glass and silicon wafers.

The present invention provides an improved method for the direct detection of the amplification product and hence target sequence, without any separation or further manipulation and with higher specificity using molecular energy transfer. Further, the invention also enables detection of amplification product without opening the reaction vessel. In this embodiment the close tube format greatly reduces the carryover contamination with amplification products, one of the few hindrances for wide spread acceptance of PCR in many applications. The method is also a very fast and efficient method.

The method of invention also allows real time measurement, high through put of samples and total automation. The method of invention also allows higher specificity and reliability of detection, greatly reduces the false positive and false negative problem associated with PCR detection the other major problem

associated with the general acceptance of the PCR in many applications. The method is also applicable for heterogenous phase nucleic acid amplification and detection in addition to the homogeneous phase amplification and detection. Further the method is also applicable for detection of single nucleotide polymorphism, deletion and addition mutations, heterozygous mutations, repeat length mutations of small repeat, methylated DNA.

The present invention relates to improvement in kits and labeled oligonucleotide amplification primer sets for the detection and or measurement of polynucleotide nucleic acid amplification products, polynucleotide/nucleic acid target sequence in sample. Such kits may be research kits, diagnostic kits or otherwise, where the nucleic acid target being amplified is correlated with the presence or absence of a disease or disorder of human, plant or otherwise, presence or absence of an infectious agent of human, plant or otherwise, presence or absence of specific genetic trait or marker of human, plant or otherwise.

Detailed Description of the Invention

Primer / dimers (homo and hetero) are non – specific amplification products formed because of same primer sitting across each other near 3' end and getting extended by polymerase (homodimer) or two primers sitting across each other near their 3' end and getting extended by polymerase (heterodimer). Only heterodimer formation between the forward and reverse primer can interfere in the signal measurement, when two labeled primers labeled separately with donor or acceptor is used. However in case of acceptor labeled oligonucleotide primer, being provided in hair – pin quenched configuration, can result in increase in noise. This noise can be corrected spectroscopically by additionally exciting the acceptor with acceptor specific excitation radiation or light. Nowadays, very good softwares for analysis of primers for primer dimer are available. So carefully chosen primers will not give primer dimer product. Moreover, synthesized primers can easily be checked for primer dimer formation by carrying amplification reaction in absence of template nucleic acid. Amplification of a product of the size close to that of primer dimer being efficient will require in general lesser concentration of primers. Again hair – pin primers being more efficient than linear primers in amplification reaction, will be needed in amplification reaction in lesser

quantity. Primer dimer formation can also be avoided by using less quantity of primer and enzyme. Labeling of oligonucleotide primers, near their 3'ends with MET / FRET moiety reduce primer dimer formation. Use of hair-pin primers with stem length of 7-9 nucleotide pairs and reduced annealing time reduce primer dimer formation.

The present invention relates to the detection and, or quantitation of nucleic acid or polynucleotide target sequence / sequences or nucleic acid/polynucleotide amplification product / products through molecular energy transfer (MET) between two rightly configured oligonucleotides (hair – pin and / or linear or other) labeled separately at or near their 3' end with a donor or an acceptor moiety of a MET pair so that it does not affect amplification or primer extension, where the acceptor emits the transferred energy in the form of radiation / light at its characteristic emission wavelengths or as heat, i.e. the acceptor moiety is a fluorophore or a quencher. One of the above two labeled oligonucleotides is an amplification primer, while the other one is the second amplification primer.

In one case both the oligonucleotides, each labeled separately with a donor MET pair moiety or an acceptor MET pair moiety, are the two primers of the PCR amplification reaction, which get incorporated into the two opposite strands of the amplification product respectively; and the two are so configured (proximal) that MET can take place between the donor and the acceptor MET moieties thus incorporated.

In another case one of the oligonucleotide primers labeled with a MET moiety gets incorporated into one of the two strands of the amplification product and the second oligonucleotide labeled with the other moiety of the MET pair hybridizes to this strand bringing the two moieties of the MET pair in appropriate proximity resulting in MET between the two partners of the MET pair.

The detection and / or quantitation of the amplification or amplification product formation is not on the basis of incorporation of a MET primer (primer containing a donor MET moiety and a quencher, or a donor and acceptor MET pair) into the PCR amplification product, but rather on the basis of the MET between the donor

and the acceptor MET moieties brought into proximity so as to MET to occur through incorporation of two separately labeled amplification primers (labeled separately with a donor MET moiety or an acceptor MET moiety) into the two separate complementary strands of the PCR product; and primarily measurement of the sensitized emission energy from the acceptor MET moiety and optionally that of the donor moiety and in addition optional excitation of the acceptor by its specific excitation light or radiation and measurement of its emission when the acceptor is provided in quenched configuration for accurate estimation of the target sequence or amplification product.

The nucleic acid amplification oligonucleotides of the invention utilize the principle of MET between a donor moiety and an acceptor moiety of a MET pair and measurement of sensitized emission. In a preferred embodiment, the MET is fluorescence resonance energy transfer (FRET), where the donor moiety is a fluorophore and the acceptor moiety is also a fluorophore. On excitation the donor fluorophore emits fluorescent energy of wavelengths different from the excitation wavelength. The fluorescent energy emitted by the donor fluorophore is absorbed by the acceptor fluorophore, which in turn releases the absorbed energy by emitting fluorescence at wavelengths different from that of the donor fluorophore. The emission of the acceptor is measured and optionally that of the donor to assess the progress of the amplification reaction. Further optional additional measurement of the emission from the acceptor after exciting the same with its specific excitation wavelength will enable accurate estimation of the amplification product or the target sequence.

The method also relate to an improvement in signal to noise ratio by providing the acceptor moiety quenched with a quencher in a hair – pin configuration of the labeled oligonucleotide so that the excitation of the acceptor moiety by donor excitation radiation / light is minimized resulting in low background and the acceptor emits its characteristic radiation / light in the open configuration of the acceptor – quencher labeled oligonucleotide when incorporated into the amplification product or hybridized to the labeled strand of the amplification product due to partial excitation of the acceptor by the donor excitation radiation / light and the non – radiative transfer of the energy from the excited donor to the

acceptor thus resulting in higher signal to noise ratio (all prior art methods depend on signal generation by emission from the fluorophore in the open configuration of the fluorophore – quencher labeled oligonucleotide only).

The invention also provides a fast, sensitive and more reliable simple homogeneous phase method for the detection of nucleic acid targets or nucleic acid amplification products. The method does not require further manipulation of any sort after the amplification reaction is set and also the method does not require any special amplification protocol like hot start PCR. Further the method is free of many other drawbacks of the prior art such as personal errors, sample type and quality, requirement of skilled workers etc.

It is adaptable and related to close tube format thus greatly reducing possibility of carryover contamination of amplification product. The method also relates to end point measurement as well as measurement in real time and can be automated increasing throughput. Further the method again relates to very high throughput because of the shorter cycling time. The method is adaptable for many methods of nucleic acid amplification including polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), allele specific – PCR, DNA methylation status-PCR, nested PCR, in situ PCR and also relates to detection of different types of mutation and splice variant mRNA transcripts and very high throughput real time RNA expression profiling.

The method also relates to heterogeneous phase detection, where one of the amplification oligonucleotide is covalently attached at its 5' end to a solid surface through a suitable linker / spacer and all other components of the amplification reaction are in liquid phase in contact with the solid surface. The solid surface is non-porous and transparent or translucent and can be glass, silicon wafer (preferably) or plastics such as polystyrene, polypropylene, polyethylene, dextran and the like.

Analytes to be detected by the detection method of this invention are polynucleotides, which may be present in any biological or non-biological sample, such as clinical samples, for example blood, urine, sputum, saliva, feces, pus,

semen, serum, other tissue samples, culture media, fermentation broth and the like. If necessary the analyte may be pre-extracted or purified by known methods of nucleic acid purification and extraction.

In a preferred embodiment either one of the amplification oligonucleotide primers or both the amplification oligonucleotide primers are hair – pin primers. These hair – pin primers are complementary to the target sequence at the 3' – end while last five to nine bases at the 5' end of the said primers may or may not be fully or partly complementary to the target sequence, but are fully complementary to 3' end or near 3' end of the said primers resulting in a hair – pin stem structure.

In the case of both the amplification primers being hair – pin primers one of the two amplification oligonucleotide primers is labeled with a donor MET pair moiety at or near its 3' end preferably near 3'end, while the other amplification oligonucleotide primer is labeled with an acceptor MET pair moiety at or near its 3' end preferably near 3' end and so labeled that it does not affect amplification or primer extension. The 5' ends of both the amplification oligonucleotide primers are labeled with a quencher / quenchers, which absorb the energy emitted by both the donor MET moiety and the acceptor MET moiety, in such a way that in both cases, the quencher / quenchers come very close to both the donor and the acceptor moieties in the hair – pin stem structures resulting in quenching of the emission of the donor as well as the acceptor MET moiety. This should result in reduction in radiation / emission background at the emission wavelength of the acceptor by minimizing the contribution from the emission of the donor moiety in the above wavelength if any; and emission from the acceptor due to the excitation of the same by the radiation used for the excitation of the donor. The quencher is preferably a non – radiative quencher. When not incorporated into the amplification product the above primers will remain in quenched closed hair – pin configuration resulting in almost negligible background but when incorporated into the amplification product are no more quenched and the acceptor moiety can absorb the energy emitted by the excitation of the donor resulting in MET and characteristic emission of the acceptor, which can be measured. This negligible background thus achieved would help detection of extremely low copy number polynucleotide target / targets in a sample.

In the case of one of the amplification primers being hair – pin primer of the invention, the hair – pin oligonucleotide amplification primer is labeled with an acceptor MET pair moiety at or near its 3' end preferably near 3'end, so labeled that it does not affect amplification or primer extension, and with a quencher, which absorbs the energy emitted by the acceptor at or near its 5' end, in such a way that the acceptor MET moiety and the quencher come very close to each other in the stem structure of the said hair – pin primer and there is maximum quenching in the emission of the acceptor MET moiety by the quencher in the hair – pin configuration of the above said primer. The quencher is preferably a non – radiative quencher. The second amplification oligonucleotide primer is a linear primer labeled near its 3' end with the donor MET pair moiety so labeled that it does not affect amplification or primer extension. The acceptor labeled hair – pin primer remains quenched when not incorporated into the amplification product but when incorporated, is no more quenched and the acceptor moiety can absorb the energy emitted by the donor moiety incorporated into the other strand of the amplification product resulting in MET and characteristic emission from it, which can be measured. The hair – pin configuration of the primer labeled with the acceptor moiety results in reduced background at the emission wavelength of the same when the above said primer is not incorporated into the amplification product by quenching of the emission from the acceptor due to the excitation of the same by the radiation used for the excitation of the donor. This would help detection and / or quantitation of very low copy number polynucleotide target / targets in a sample.

In both cases MET will occur only when both the amplification primers one having the donor MET moiety and the other having the acceptor moiety, get incorporated into the two complementary strands of the right amplification product and in right configuration as designed for MET to occur. The detection and / or quantitation of the target sequence is based on the measurement of emission from the acceptor MET moiety and or optionally measurement of reduction in donor emission.

Moreover, above hair – pin primers of loop and stem structure (strained) will give better specificity of primer or primers binding to the target during amplification

and placing of quencher close to the acceptor moiety in the hair – pin configuration of the amplification primer labeled with the acceptor moiety near its 3' end will drastically reduce or eliminate the background due to the excitation of the acceptor MET moiety by the light or radiation used to excite the donor MET moiety.

In another embodiment both the oligonucleotide amplification primers, which are linear non-duplex primers are labeled at or near the 3' end preferably near 3'end of them either with a donor or an acceptor MET moiety. The two primers are so labeled that amplification reaction is not affected and so designed that when the two primers get incorporated into the two strands of the right amplification product, the donor and the acceptor moiety are placed in correct proximity so as to result in MET between the two moieties. The emission of the acceptor and optionally that of the donor is measured. In an extension of the same the acceptor is a non – radiative quencher for the donor and signal is measured by measuring reduction in donor emission.

In another preferred embodiment of the invention one of the amplification oligonucleotide primers is in hair – pin configuration, labeled at or near its 3' end preferably near 3'end, with an acceptor MET pair moiety, while the distant 5' end is attached to another five to nine base oligonucleotide, which may or may not be fully or partly complementary to the target sequence but fully complementary to the 3' end of the said amplification primer to form stem structure of the said hair – pin, and the above five to nine base oligonucleotide carries a preferably non – radiative quencher absorbing in the entire visible region, or in the emission range of the acceptor MET moiety at right configuration in order to maximize quenching of the acceptor MET moiety in case of excitation by donor excitation radiation. Detection and / or measurement of the amplification product are based on the measurement of emission from the acceptor MET moiety and quenching or reduction in the donor emission. Use of hair – pin primer with acceptor and quencher will reduce drastically or eliminate the background emission.

In still another preferred embodiment of the invention one of the amplification oligonucleotide primers is labeled at or near its 3' end preferably near 3'end with

a donor MET pair moiety so labeled that it does not affect amplification or primer extension, and can be a linear or a hair – pin primer, The above said labeled linear amplification primer is fully complementary to a specific region of a strand of the target sequence for the amplification of which the primer is designed. The above said labeled primer in hair – pin configuration is the above said linear primer at the 5' end of which is added additional five to nine bases which may or may not be partly or fully complementary to the target sequence but fully complementary to the five to nine bases at or near the 3' end of the above said primer and at the end of these additional five to nine bases, i.e., extreme 5' – end of the hair – pin primer or near it is attached a quencher which absorbs emission in the entire visible spectral region (for eg. DABCYL or its derivative) or in the emission range of the donor MET moiety to result in maximum quenching of the donor emission in closed hair – pin configuration.

In yet another preferred embodiment of invention, the method provides a higher signal to noise ratio in comparison to other prior art methods and accurate estimation of the target sequence or amplification product. MET will occur when the labeled amplification primers get incorporated into the amplification product and in right location designed for MET to occur. Detection and / or measurement of the amplification product is based on the measurement of the emission from the acceptor and optionally measurement of donor emission. The labeled hair – pin primer remains in closed quenched configuration when not incorporated into the amplification product but in open configuration when incorporated into the amplification product thus allowing the excitation of the donor MET moiety by the excitation radiation and characteristic emission from the donor, which can be transferred to the acceptor MET moiety in its open configuration. The use of hair – pin primers result in very low background and hence allows higher detection sensitivity.

In this preferred embodiment a higher signal to noise ratio and accurate estimate of target sequence is provided. The first oligonucleotide of the invention labeled at or near its 3' end with the acceptor MET moiety of the donor – acceptor MET pair, is of hair – pin configuration, in one strand of the stem structure of which the acceptor MET moiety is attached and in the complementary strand of the stem a

quencher is attached; the acceptor and quencher are so configured that emission of the acceptor moiety remains maximum quenched in the closed configuration of the above hair – pin oligonucleotide. The above oligonucleotide is so labeled that when it is an amplification primer, the amplification reaction or the primer extension is not affected. Because of the acceptor MET moiety remaining quenched when the above said oligonucleotide remains free in solution without being incorporated into or hybridized to the amplification product, the excitation of the above said acceptor MET moiety by the donor excitation radiation / light is negligible resulting in low background emission from the acceptor MET moiety. On the other hand when the above said labeled hair – pin oligonucleotide is either incorporated into or hybridized to the amplification product remains in open configuration separating the acceptor MET moiety and the quencher. On illumination by donor excitation radiation or light the acceptor-MET moiety gets partially excited, emitting its characteristic emission. The ratio of this emitted energy (i.e. signal) to the background would be the same as that if it was illuminated with the radiation / light characteristic for acceptor excitation. Further during MET between the donor moiety on the second oligonucleotide, which is again a primer, the excitation energy of the donor due to excitation of same with the donor excitation radiation (light) is transferred to the acceptor MET moiety, which in turn emits absorbed energy as its characteristic emission, thus augmenting or increasing the signal (acceptor emission). As a result the signal to background / noise ratio increase resulting in higher sensitivity of detection.

The signal measurement is mainly based on measurement of the increase in acceptor emission after exciting the donor with the donor specific excitation radiation / light. This will be sum total of emission due to specific and non – specific product plus molecular energy transfer. Optionally measurement of the quenching of the donor emission or reduction of donor lifetime will allow cross checking of the result. Further, in addition to the above acceptor emission measurement, if the acceptor is excited with the acceptor specific radiation or light the increase in acceptor emission will give the measure of the total emission i.e., the emission from specific and non – specific product formation. This emission measurement can be easily normalized for excitation by the donor specific excitation radiation. Thus subtraction of this normalized acceptor

emission from acceptor emission utilizing donor specific radiation will give the measurement of the MET, which will be the correct measurement of the specific amplification product.

In yet another embodiment the donor and or the acceptor moieties on the labeled oligonucleotides primer / primers are quenched preferably with a non-radiative quencher which absorbs, light in the entire visible region or the spectral emission region of the donor and / or the acceptor, and which is part of a second oligonucleotide partly or fully complementary to the target sequence but fully complementary to the last five to nine bases at or near the 3' end of the labeled oligonucleotides, and is attached to the 5' end of the above said oligonucleotide through a short linker at the 3' end of the above said second oligonucleotide. The above said second oligonucleotide so designed that the said labeled oligonucleotides remain quenched when not incorporated into the amplification product and remain in open configuration when the above said labeled oligonucleotides are incorporated into the amplification product. The above linker is either a third oligonucleotide of length between two bases to twelve bases, which may or may not be fully or partly complementary to the target sequence or a short organic linker. The MET moieties are placed near the 3' end of the labeled oligonucleotide and the quencher (like DABCYL or its derivative which absorbs in the entire visible region or any other quencher which absorbs in the spectral emission region of the donor or the acceptor MET moiety) is placed at the 5' – end of the second oligonucleotide; in such a way that the donor and / or the acceptor MET moieties are in close proximity to the quencher in order that quenching of emission from the donor or acceptor can take place in closed configuration, i.e, when the labeled primer / primers are not incorporated into the amplification product. MET between the donor and the acceptor moieties occurs when the two labeled primers get incorporated into the specific amplification product. The emission of the acceptor MET moiety and optionally that of the donor is measured to monitor the amplification process or detection and / or quantitation of the amplification product.

In still another embodiment the donor or the acceptor moieties on the oligonucleotide primers are provided quenched with the help of additional two

more oligonucleotides labeled at their 5' ends with suitable quencher for donor or acceptor respectively and complementary to the oligonucleotide primers, when not incorporated into the amplification product. The signals are generated from the separation of the donor and acceptor labeled oligonucleotide primers from the quencher labeled complementary oligonucleotides as the above donor and acceptor labeled primers are incorporated into the amplification product.

In still further embodiment, the invention provides heterogeneous phase nucleic acid amplification (PCR / LCR) assay in addition to the homogeneous phase assay. In heterogeneous phase assay one of the amplification primers (labeled or unlabeled) is fixed or attached at its 5' end to a non-porous solid support through a linker / spacer (preferably water soluble or hydrophilic) while the other primer (labeled or unlabeled) are in solution phase in contact with the non-porous solid support (glass, silicon wafer, polypropylene, polystyrene, nylon, cellulose and others) along with the other reagents required for the amplification. The solid surface can be flat like a glass slide or plastic laminate, and the like; or curved like a thin walled plastic tube or cuvette, a well or a microtiter plate or a silicon wafer microtiter plate and the like.

In a further embodiment, of the invention provides two or four linear and / or hair – pin oligonucleotides primers (non – duplex), which are separately labeled with donor or acceptor MET moieties such that MET will occur only when the respective primers are ligated in Ligase Chain Reaction (LCR). In this embodiment the oligonucleotides are in hair – pin configuration, where near the one end of the stem there is a donor or an acceptor MET moiety attached and at the other end opposite to the MET pair moiety there is a non – radiative quencher like DABCYL, so that the emission of the MET moiety of the unligated oligonucleotides are quenched.

In yet another embodiment the invention provides real time measurement and a close tube format in which no further manipulation or opening of the reaction tube is required once the amplification reaction is set up. This will greatly reduce the carryover contamination problem associated with nucleic acid amplification, which is one of the major hindrances for routine PCR analyses.

The invention provides a method for detection and measurement of a nucleic acid target and nucleic acid amplification product through an amplification reaction comprising;

The sample to be analyzed consisted of nucleic acids, and all reagents required for amplification in a container (reaction vessel) which can be a well, tube, or a cuvette and is transparent or translucent for the aid of measurement, at least two (or more) oligonucleotides, a first one of said oligonucleotides of a sequence complementary to a preselected sequence of the target, that may be present in said sample, that is labeled with a first moiety so labeled that the amplification reaction or the extension of the primer can take place, from the group consisting of a donor moiety and an acceptor moiety of a molecular energy transfer pair, and which gets incorporated into the amplification product, in association with a second of said oligonucleotides being a pair of primers adapted for use in said amplification reaction such that said primers are incorporated into amplified product of said amplification reaction, when said target sequence is present in the sample; in one embodiment the said second oligonucleotide is labeled with a second moiety so labeled that the amplification reaction or primer extension can take place, selected from the group consisting of said donor moiety and said acceptor moiety, said second moiety being the member of said group that is not said first moiety, wherein said primer labeled with said first moiety and said oligonucleotide labeled with said second moiety are configured so as to be incorporated into the two strands of the said amplification product wherein the donor moiety emits energy of one or more particular wavelengths when excited, and the acceptor moiety absorbs energy at one or more particular wavelengths emitted and emits energy at one or more wavelengths, different from that of the donor

(i) Conducting the amplification reaction.

(ii) Stimulating light emission from the donor moiety

(iii) Detecting or measuring energy emitted by said acceptor moiety, and optionally that of the said donor moiety and in addition exciting the acceptor moiety with its characteristic excitation radiation or light and

measuring the acceptor emission for very accurate measurement of the target sequence or amplification product.

The nucleic acids in the sample may be DNA or RNA (modified or unmodified) purified or unpurified.

The pair of primers, i.e. one forward primer and one reverse primer, for use in PCR or RT – PCR or other PCR and nucleic acid amplification reactions consists of oligonucleotide primers that are complementary to the two different complementary nucleic acid strands of the target nucleic acid, such that the extension product of one primer towards the other primer generated by nucleic acid polymerase, can serve as template for the extension of the other primer. The nucleic acid amplification product is the content of nucleic acid in the sample between and including the two primer sequences. Nucleic acids that are "complementary" can be perfectly or imperfectly complementary, as long as the desired property resulting from complementarities, i.e. ability to hybridize is not lost.

In a specific embodiment, the invention provides a method for the detection and quantitation of a nucleic acid amplification product in amplification reaction comprising (a) contacting a sample comprising nucleic acid with two oligonucleotide primers, said oligonucleotide primers being adapted for use in said amplification reaction such that the said primers are incorporated into an amplified product of said amplification reaction, when a preselected target sequence is present in the sample; both the primers are individually labeled with either a donor moiety or an acceptor moiety in a way such that amplification reaction or primer extension can take place, where the acceptor moiety emits energy at one or more wavelengths different from that of the donor or as heat as the case may be. Both the primers can be linear, or one linear and one hair - pin, the hair- pin one containing the acceptor moiety, near the 3' end and a quencher for the acceptor near the 5'- end or both hair - pin each containing a donor or an acceptor moiety near the 3' end and respective quenchers near the 5'-ends of both, quenchers are different from the donor and the acceptor moieties. (b) carrying out of the amplification reaction, (c) stimulating of emission from said

donor moiety; and (d) detection or quantitation of the emission from the acceptor moiety and optionally the emission of the donor and in addition excitation of the acceptor with its characteristic excitation radiation or light and measuring the emission from the acceptor for very precise or accurate measurement of the target sequences or amplification product.

The present invention also provides a method for the detection or measurement of a product of a nucleic acid amplification reaction involving (a) contacting a sample comprised of nucleic acids with two oligonucleotide primers designed for use in said amplification reaction. At least one of the said oligonucleotide primers being labeled with a first moiety from the group consisting of a donor moiety and an acceptor moiety and so labeled that it does not affect amplification (or primer extension), and either of linear or hair – pin configuration; in case of hair-pin configuration there also being a quencher near the 5'-end of the said primer and the first moiety is the acceptor so configured that the first moiety remains quenched in closed hair-pin configuration of the said primer when not incorporated into the amplification product and remains unquenched when incorporated into the amplification product; such that both the primers get incorporated into the amplification product of the said amplification reaction, when a preselected target sequence is present in the sample, and wherein at least a different oligonucleotide, complementary to a preselected sequence of the strand of the said amplification product into which the said labeled primer has been incorporated, labeled with the second moiety selected from the group consisting of the said donor and said acceptor moiety; said second moiety being the member of said group that is not said first moiety and either of linear or hair-pin configuration, in case of hair - pin configuration there being also a quencher like DABCYL at or near the 5' end of the said second oligonucleotide so configured that the second moiety remains quenched in a closed hair-pin configuration of it , when it is not hybridized but the second moiety remains unquenched in the open configuration of the said second oligonucleotide when it hybridizes to the said strand of the amplification product thus allowing MET to occur between the said donor moiety and the said acceptor moiety (b) conducting amplification reaction (c) stimulating light emission from said donor moiety; and (d) detecting or measuring the energy emitted by the acceptor moiety and optionally the energy

emitted by the donor and in addition excitation of the acceptor with its characteristic excitation radiation or light and measuring the emission from the acceptor for very accurate estimation of the target sequence or amplification product.

The present invention provides a method for the direct detection of the amplification product. This method is a an improvement over the methods already available in the prior art by removing the sources of error and improving the sensitivity of detection maintaining a high specificity. It permits detection of amplification product without any separation, hence permitting detection without opening the tube, i.e. in close tube format thus reducing greatly the crossover contamination problem with amplification product that has slowed down the acceptance of PCR for routine analyses. The close tube format and the size of amplification product of the invention also enable very high throughput sample analysis and automation. The present invention also relates to kits for the detection and or measurement of nucleic acid amplification product, or products or detection and / or measurement of nucleic said target sequence or sequences.

The oligonucleotides for use in the invention can be of any suitable size, preferably in the range of 10 to 50 nucleotides, and more preferably between 15 to 40 nucleotides.

The oligonucleotide can be DNA or RNA or chimeric mixtures or derivatives or modified versions there of, so long as it is capable of priming the amplification reactions or hybridizing the desired amplification product. In addition to being labeled with a MET moiety, the oligonucleotide can be modified at the base moiety, sugar moiety or phosphate backbone, and may include other appending groups including linker or spacer arms, or labels so long as it is still capable of priming the amplification reaction or hybridizing with the amplification product as a probe.

For example the oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5- bromo-uracil, 5, fluoro-uracil, 5- chloro-uracil, 5-iodo-uracil, hypoxanthine, xanthine, 4-

acetylcytosine, 5-(carboxy hydroxy methyl) uracil, 5-carboxymethyl aminomethyl-2 thiouridine, 5'-carboxy methyl aminomethyl uracil, dihydrouracil, □-D-guanosine, inosine, N6-isopentynyladenine, 1-methyl guanine, 1-methyl inosine, 2,2-dimethylguanine, 2-methyl adenine, 2-methyl guanine, 3-methyl cytosine, 5-methyl cytosine, N6-adenosine, 7-methyl guanine, 5-methyl aminomethyl uracil, 5-methoxy aminomethyl-2 thio uracil, □-D-mannosyl queosine, 5-methoxy carboxymethyl uracil, 5-methoxy uracil, 2-methyl thio-N6-isopentenyladenine, uracil 5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thio-uracil, 4-thiouracil, 5-methyl uracil, uracil-5-methyl ester, uracil 5-oxyacetic acid (v) 5' methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp)3W, and 2,6 diaminopurine. The oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinase, 2-fluoroarabinose, xylulose and hexose.

In addition, the oligonucleotide may comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphoradiamidate, a methyl phosphonate, an alkyl phosphotriester, formacetal, peptide nucleic acid or analong thereof.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, for e.g., by de novo chemical synthesis using an automated DNA synthesizer (such as commercially available machines from Biosearch, Applied Biosystems and many other suppliers using phosphotriester chemistry) or by cleavage of a larger nucleic acid fragment using non-specific nucleic acid cleaving chemicals or enzymes or site specific restriction endonucleases. Alternatively those can be obtained from commercial suppliers.

Phosphorothioate oligonucleotides may be synthesized by the method of Stein et.al Nucl. Acids Res. (1988, 16, 3209), methylphosphonate oligonucleotides can be synthesiszed by the method of Sarin et.al. (Proc. Natl. Acad. Sci. USA 1988, 85, 7448-7451) etc.

The oligonucleotides can be purified by any method known in the art, including extraction, gel permeation chromatography, gel electrophoresis and HPLC purification. The concentration of the oligonucleotide can be measured by measuring optical density at 260 nm in a spectrophotometer. Purity of the oligonucleotide can be determined by polyacrylamide gel electrophoresis or HPLC as known in the art.

The oligonucleotides of the invention may be labeled with the donor and acceptor moieties as well as the quencher during chemical synthesis or by attachment after synthesis by methods known in the art. Both the donor and the acceptor moieties are fluorophores, europium chelates or other entities. Suitable moieties that can be selected as donor or acceptor in FRET pairs, as well as the quencher moieties are given in the table. Selection of the donor and acceptor of FRET pair is decided on the basis of the spectral overlap of the two fluorophores and molar extinction coefficient of absorption of radiation or light and quantum yield as known in the art.

Suitable moities that can be selected as donor or acceptors in FRET pairs.
4 – acetamido – 4' – isothiocyanatostilbene – 2,2' disulfonic acid
acridine
acridine isothiocyanate
5 – (2' – aminoethyl) aminonaphthalene - 1 – sulfonic acid (EDANS)
4 – amino – N – 3 – vinylsulfonyl) phenylnaphthalimide – 3,5 disulfonate(lucifer yellow vs)
N-(1-anilino-1-naphthyl) maleimide
anthranilamide
brilliant yellow
coumarin
7-amino-4-methylcoumarin (amc, coumarin 120)
7-amino-4-trifluoromethylcoumarin (coumarin 151)
cyanosine
cyanine-3
cyanine-5
4', 6-diaminidino-2-phenylindole (DAPI)

5',5" – dibromopyrogallol – sulfonaphthalein (Bromopyrogallol Red)

7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin

diethylenetriamine pentaacetate

4,4' –diisothiocyanatodihydro-stilbene –2, 2' –disulfonic acid

4,4' –diisothiocyanatostilbene –2, 2' –disulfonic acid

5-dimethylamino1 Naphthalene-1-sulfonylchloride (DNS, dansyl chloride)

4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL)

4-dimethylaminophenylazophenyl –4' –isothiocyanate (DABITC)

eosin

eosin isothiocyanate

erythrosin and derivatives;

erythrosin b

erythrosin isothiocyanate

ethidium

fluorescein

fluorescein isothiocyanate

5- carboxyfluorescein (5-FAM)

6-carboxyfluorescein (6-FAM)

5-(4,6 –dichlorotriazin –2 –yl) aminofluorescein (DTAF)

2'7'–dimethoxy –4'5–dichloro–6-carboxyfluorescein (JOE)

fluorescamine

IR144

IR1446

Malachite green isothiocyanate

4 –methylumbelliferone

ortho cresolnaphthalein

nitrotyrosine

pararosaniline

Phenol Red

B-phycoerythrin

pyrene

pyrene butyrate

succinimidyl 1 pyrene butyrate

Reactive Red 4 (cibacron .RTM .Brilliant Red 3B –A)

6-carboxy –X–rhodamine (ROX)
6-carboxyrhodamine (R6G)
lissamine rhodamine B sufonyl chloride
rhodamine(Rhod)
rhodamine B
rhodamine 123
rhodamine x isothyiocyanate
sulforhodamine b
sulforhodamine 101
sulfonyl chloride derivative of sulforhodamine 101
(Texas Red)
N,N,N' ,N'-tetramethyl-6-carboxyrhodamine (TAMRA)
tetramethyl rhodamine
tetramethyl rhodamine isothiocyanate(TRITC)
riboflavin
rosolic acid
terbium chelate derivatives
europium chelate derivatives

Preferably, the oligonucleotides used in the invention are selected from the following sequences:

1). 5'-GGG GTA CTA CAG CGC CCT GA – 3'
2). 5'-GGG GTA CTA CAG CGC CCT GA –3'
|
FAM

3). 5'-GTC CTG GAA GAT GGC CAT GG –3'
4). 5'-GTC CTG GAA GAT GGC CAT GG –3'
|
JOE

5). 5'-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

6). 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG -3'

JOE

7). 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

FAM

8). 5'-GCT CAT GGC GCC TGC CTG G –3'

DABCYL

9). 5'-DABCYL-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

JOE

10).5'-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

11).5'-DABCYL-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

FAM

Hair – pin Oligonucleotide suitable for use in the method:-

The present invention provides oligonucleotide primer / primers that form a hair – pin structure. The hair – pin oligonucleotide (primer) are of the length between 15 to 60 nucleotides or more specifically between 20 – 45 nucleotides complementary to one or the other strand of the target sequence except the last five to nine nucleotides at the 5' end of the same oligonucleotide, which may or may not be partly or fully complementary to the target sequence but fully complementary to the five to nine nucleotides at or near the 3' end of the above oligonucleotide so that a hair – pin stem structure of five to nine nucleotides can be formed. The above said hair – pin oligonucleotide primer / primers are labeled at or near the 3' end preferably near 3'end (within fifteen nucleotides from 3' end) when the oligonucleotide / oligonucleotides are primers labeled with an acceptor

MET moiety or a donor MET moiety of a MET pair and so labeled that it does not affect the amplification reaction or primer extension. At the 5' end of the above oligonucleotide is covalently attached a quencher which absorbs in the spectral emission region of the above said donor and / or acceptor MET moieties or in the entire visible spectral region and emits the absorbed energy preferably in the form of heat (for e.g. DABCYL or its analog) or as emission at a wavelengths different from that of the donor and / or the acceptor. The acceptor or the donor MET moiety and the quencher are either on the two complementary strands of the stem structure separated by a distance of less than one nucleotide or 2 – 5 nucleotides on the complementary strands of the stem structure depending on the MET moieties when the above said oligonucleotides are in their closed hair – pin configuration. These doubly labeled oligonucleotides are in the closed quenched hair – pin configuration when used as primer / primers and not incorporated into the amplification product. When used as primer / primers and incorporated into the amplification product, the above doubly labeled hair – pin oligonucleotide / oligonucleotides (as the case may be) will be in open configuration so that the donor or the acceptor MET moieties are no more quenched by the quencher; the donor can be excited by the donor excitation radiation and the acceptor can absorb the energy emitted by the donor when in proximity for MET to occur, and can emit its characteristic emission. Information on the design of hair – pin primers is known in the prior art.

Details of the invention, its objects and advantages are explained hereunder in greater detail in relation to the non-limiting exemplary illustration of the method of the invention :

EX-1 THE DETECTION AND/OR QUANTITATION OF A NUCLEIC ACID TARGET USING TARGET AMPLICATION

In the detection and/or quantitation of a nucleic acid target using target amplification, the main artifacts are non-specific products formation and primer dimer product formation utilizing non-separation type of method for measurement. Sometimes primer dimer formations interfere with the measurement or lead to amplification failure. For this reason non-specific

production formation and primer dimer formation should be minimized preferably eliminated. By optimizing the reaction conditions the non-specific product formation can be avoided to large extent but difficult to eliminate. Further high stringency PCR for avoiding non-specific product formation many times result in PCR failure. Non-specific product formation and primer dimer formation are the two main factors for lowering of the signal to noise ratio in non-separation based nucleic acid target detection and/or quantitation methods like FRET based, fluorescence based, DNA intercalating dye fluorescence based, etc. , For this reason there are attempts to increase the quenching efficiency of FRET based (fluorophore - quencher) methods to increase signal to noise ratio, Nature Biotechnol (2001) 19, 365-370. However, a small percentage of non-specific product formation or primer dimer formation, will bring down the signal to noise ratio to a large extent.

In our effort to develop primarily a sensitized emission (FRET) based homogeneous phase high specificity and high sensitivity detection method, we attempted a strategy where an amplification product of the size close to the size of primer dimer product (size of the forward primer plus the size of the reverse primer plus 0-25 bases) and primers labeled internally with fluorophore or quencher need to be used and primer dimer formation should be eliminated. In PCR amplification reaction, primer dimer formation interfere in PCR amplification reaction and in many occasions lead to PCR failure. In our approach use of an amplification product of the size close to that of primer dimer for nucleic acid target analysis was expected to gain advantage from the presumption that size of the primer dimer can have amplification advantage and if this could be utilised favourably in nucleic acid target amplification.

Primer dimers are assumed to be efficient templates for nucleic acid amplication. Even if a primer dimer product is formed at an early stage of amplification, the specific product being of the size close to that of primer dimer, primer dimer product cannot overtake specific product, on the other hand specific product being equally efficient template and present in good number initially will overtake primer dimer product resulting in formation of specific amplification product only and very little primer dimer product in presence of target DNA.

On the basis of the assumption that if an amplification product of the size close to that of primer dimer can be an efficient template for PCR amplification, it will easily overtake any non-specific product formed at any stage of amplification reaction. Chances of formation of a non-specific amplification product of the size close to that of a primer dimer product that can compete with specific amplification product is very rare. Hence chances of formation of any non-specific amplification product, is expected to be rare.

If primer dimer formation during PCR can be reduced to negligible level or eliminated use of an amplification product of the size close to that of primer dimer for monitoring nucleic acid amplification can be a good proposition. It is known that use of lesser quantity of primers and Taq DNA polymerase in PCR reduces primer dimer formation. Elimination of primer dimer formation can only be achieved by designing good primer pairs. A combination of good or reasonably good primer pair, appropriate primer and enzyme concentration can fully eliminate primer dimer formation. It is also easy to check primer pairs for primer dimer formation. Primer pairs can be checked for primer dimer formation by carrying out PCR without any template DNA.

Amplification of an amplification product of the size close to that of primer dimer is likely to give higher yield of the amplification product resulting in higher signal to noise ratio.

FRET based method can be employed for detection resulting in still higher signal to noise ratio and higher specificity.

Many other signal generation method other than FRET i.e. intercalating dye fluorescence, and heterogenous phase signal generation like ELISA, radioisotope can be used.

Single tube, real time monitoring of PCR amplification or PCR amplification product, high throughput PCR analysis, and real time quantitative RNA expression profiling can be carried out.

(A) In presence of template DNA specific amplification product only formed and no primer dimer formed.

In order to show that for amplification of an amplification product of the size close to that of primer dimer it is the specific amplification product and not the primer dimer that is formed during amplification reaction in presence of target DNA, primers were designed for the amplification of the 60 base pair segment (base Nos.1094-1153) and 40 base pair segment (base Nos.1114-11153) of gp63 gene of Leishmania donovani (accession No.M60048). Only specific amplification products and no primer dimer was formed, which indicates that amplification of an amplification product of the size close to that of primer dimer may be as efficient as the formation of primer dimer (fig.20). Oligonucleotide sequences 8 and 13and 10 and 13 respectively were used as amplification primers.

Primers Seq.6 and Seq.16 supposed to give an amplification product of the size 47bp. The same primer pair gives the primer dimer formation at annealing temperatures between 55-65° C. But in presence of template give mostly amplification product and very little primer dimer (fig 21A lanes 1&2)

(B) Use of an amplification product of the size close to that of primer dimer for nucleic acid amplification helps in eliminating or reducing non-specific amplification product formation.

A number of amplification primers designed from an approximately 593 bp base pair segment (base nos.560 - 1153) of gp-63 gene of Leishmania donovani were used to amplify different segments of the same in the size ranges of 36 bp to 60 bp and 544 bp to 588 bp. In case of amplification of the amplification products in the size range of 36 to 60 bp there were no product formation other than the specific product i.e. no non-specific product formation of larger size (fig.21) while in case of amplification of the amplification products in the size range of 544-588bp there was a lot of non-specific product formation (fig.22). The amplification products in the size range of 36-60 bp were of the size, which was

either the size of the forward primer plus the size of the reverse primer or, the size of the forward primer plus the size of the reverse primer plus 25 bases. This demonstrated that use of amplification primers to amplify a segment of target sequence of the size close to that of primer dimer (length of forward primer plus length of reverse primer plus zero to twenty five bases) could help in reducing or eliminating any non-specific product formation provided that the primers are designed and tested for no primer dimer formation. The same may be the result with an amplification product of size, size of the forward primer plus the size of the reverse primer minus two to three bases. Oligonucleotide sequences bearing the sequence Nos.6, 7, 8, 9, 10, 13, 14 and 15 were used in different combinations for amplifying different segment lengths of the above. In Fig.21 in case of primer pairs 6&7, 8&9, 10&13 and 8&13 used for amplification of segments of L.donovani gp63 gene, only in case of primer pair 8&13 little non-specific product formation took place because of using lower annealing temperature of 58^{0}C while primer melting temperature for primer sequence no.9 is higher than 80^{0}C. Primer 8 & 9 being hair pin primer, when instead of hair-pin primers respective linear primer pair Seq.17 & 18 (melting temperature close to 60^{0}C) were used no non-specific product formation was observed (Fig.21A lanes 8 and 9 from left)

It was also observed that in case of amplification product of the size close to that of primer dimer even lower stringency of annealing mostly did not result in any non-specific product formation and gave higher yield of amplification product. This supports that there will be less PCR failure in case amplification product of the size close to that of primer dimer is used for analysis, which would result in higher sensitivity of detection.

(C) Use of an amplification product of the size close to that of primer dimer for nucleic acid target amplification result in higher amount of amplification product :-

In order to demonstrate that amplification of an amplification product of the size close to that of primer dimer result in higher amount of amplification product, a 40bp segment (base position 1114 to 1153) and 544 bp segment (base positions

560 to 1103) of gp 63 gene of Leishmania donovani were amplified in presence of [□ ^{32} P] dATP as tracer using the amplification primers seq.nos. 10 and 13 and seq.nos.14 and 15 respectively using 50 ng of chromosomal DNA and 60°C annealing temp. for 10,15, 20, 25 and 30 cycles. The amplification products were separated by polyacrylamide gel electrophoresis and the gels were analysed in phosphor imager. The no. of [□ ^{32} P] dATP that could be incorporated into the 544 bp product is 30 times that in case of 40 bp product. There were higher amount (10-20 times) of amplification product formation in case of 40 bp product in comparison to 544 bp product (fig.nos.23 and 24 and fig nos.27-31).

(D) Use of an amplification product of the size close to that of primer dimer for nucleic acid amplification based analysis can result in higher throughput.

In order to demonstrate that use of an amplification product of the size close to that of primer dimer for nucleic acid amplification based analysis, the 40 bp segment (base position 1114 to 1153) and 64 bp segment (base position 1090-1153) of gp 63 gene of Leishmania donovani were amplified with the help of the amplification primers seq.Nos. 10 & 13 and 17 & 13 in presence of a [□ ^{32} P] dATP as tracer with shorter annealing time and without any extension step. A denaturation time of 10 seconds and annealing time of 2 seconds was sufficient for the amplification and no separate extension step was required; because of which cycling time reduced considerably. There was good amplification of specific amplification products. The products being of very small size denaturation temperature, also can be reduced which again would result in shorter cycling time. It is possible that denaturation and annealing time for cycling can be reduced further. Because of shorter cycling time and need for further very brief or no final extension step amplification of a product of the size close to that of primer dimer result in a high throughput PCR analysis. Further use of very short annealing time eliminate the formation of primer dimer and nonspecific product formation. Even the primer pair sequence no.6 & 16 which form primer dimer did not form any primer dimer in this cycling condition (Fig.25)

(E) Design of amplification primers for the amplification of an amplification product of the size close to that of primer dimer :

Designing of a good primer pair for amplification of a target nucleic acid sequence that does not give non-specific amplification product and primer dimer product formation is most difficult. For amplification of an amplification product of any suitable size, i.e. without any size restriction, good amplification primer pair that will not form primer dimer product can be designed with relatively less difficulty. But designing of good primer pair that does not give any non-specific amplification product and primer dimer product will be difficult again.

However designing of a good primer pair for amplification of target sequence for an amplification product of the size close to that of primer dimer product that does not lead to any primer dimer product formation during PCR amplification reaction is expected to be difficult again.

Design of good amplification primer pair for an amplification product of the size close to that of primer dimer will be relatively easier for nucleic acid samples with GC content of 50 percent. There are many pathogenic organisms or infectious agents containing nucleic acids of GC content more than 50 percent.. Designing of good primer pairs for amplification of any segment without any size restriction for those organisms or infectious agents as such is difficult. Hence designing of good amplification primer pair for an amplification product of the size close to that of primer dimer is again expected to be very difficult for those organisms or infectious agents.

GC content of Leishmania donovani gp63 gene is about 65 percent and the nucleotide sequence of this gene is such that designing of good PCR amplification primer is difficult. Designing of a primer pair for amplification product of the size close to that of primer dimer is expected to be still more difficult. As an extreme difficult case Leishmania donavani gp63 gene was chosen as target. A few primer pairs were designed and tested for absence of primer dimer formation and these primer pairs were so designed that those could be labeled through T base modification and at least one of them could also be

used as hair-pin primers with good stem structure stability. These primers were chosen randomly. Two of those pairs (seq.nos. 10 & 13, 17 & 18) turned out to be two good candidates. The hair-pin primers were designed for hair-pin stem and loop structures with different lengths of stem and were similarly tested for absence of primer dimer formation. Primer dimer formation using forward primer seq.no.10 and hair-pin reverse primers (seq.nos.11 & 12, forming 8 & 9 base pair stems respectively) were checked separately. There were either much less than one percent in case of 10 & 11 pair or no primer dimer formation in case of 10 & 12 in most stringent condition of amplification i.e. in absence of any template DNA (fig. 27-31).

It is demonstrated that suitable primer pairs can be designed for the amplification of a target sequence for the amplification product of the size close to that of primer dimer, i.e. the size of forward primer plus the size of the reverse primer plus zero to twenty five bases, for monitoring of an amplification reaction of a target sequence, which can be monitored by sensitized emission (FRET) as well as by many other monitoring methods.

While designing primers that do not give primer dimer product one has to avoid base complementarity among the first three to six bases at 3' end of the primers so that the two primers cannot sit on each other and get extended. The primers can be checked in many primer design software like Oligo-4, amplify 1.3, primer premier. Further we observed that primers having high GC content, stretches G and C near 3" ends formed primer dimer even though those did not have much base complementarity at their 3' end, and GC contents of approximately 50 per cent (between 45 and 55 per cent) among the six to ten bases at the 3' end of at least one primer of the primer pair helps reducing or eliminating primer dimer formation (oligo seq.nos. 10 and 13). All primers were checked for primer dimer formations at different temperatures in absence of any target sequence at primer concentration ranging from 0.18 to 0.4 μM, Taq DNA polymerase at conc.≥3 units per 100□l reaction volume whereas normally 2-2.5U is routinely used. Primer dimer formation was negligible for the primer pair oligo seq.nos. 10 and 13 and 17 and 18 at primer concentration of approximately 0.2μM.

Primers should be designed preferably from a region of approximately 50 per cent (45 - 55) GC content, lest region encompassing 10 to 20 base region with approximately 50 percent GC content so that six to eight bases at 3' ends of both the primers or at least one of the primers can have approximately 50 per cent GC content avoiding more than two G or C or combination thereof. Higher Tm of primers should be preferred. Moreover internal fluorophore labeling near 3'ends of oligonucleotide primers help avoiding primer dimer formation. The oligonucleotide primers for FRET based detection should be designed for labeling internally with fluorophore two to four nucleotides away from 3'ends for elimination of primer dimer formation.

In case of hair-pin primers, primers with different length of the stem 5 to 9 bases were checked for the stability of the structure in Zuker DNA folding analysis. Stem structures with higher thermodynamic stability were chosen and were checked for primer dimer formation. Primer dimer formation reduced with increasing length of the stem. Primers with relatively less stability of the stem structure (oligo seq.no.11 of 8 base stem) resulted in very small amount of primer dimer formation, at primer concentration of 0.4μM and almost negligible or no primer dimer formation at primer concentration of approximately 0.2μM (in combination with oligo sequence no.10) while primers with stable stem structure (oligo seq.no.12 of 9 base stem) did not give any primer dimer formation at primer concentration of approximately 0.4μM (in combination with the oligo sequence no.10). May be sluggish opening of the stem of the hair-pin oligo nucleotide primers (oligo seq.no.12) resulted in no primer dimer formation. However, both the hair-pin primers oligo seq.nos.11 and 12 resulted in specific amplification product formation in presence of the template DNA (fig nos. 26-31).

Ex.2 Internal fluorophore modification of primer of primers do not affect PCR amplification reaction :

Monitoring of amplification reaction and/or product by sensitized emission (FRET) using internally fluorophore labeled oligonucleotide primers needs to check the

efficiency of the labeled oligonucelotide primers in amplification reaction. To check if the fluorophore labeling of oligonucleotide primers could interfere in the amplification reaction fluorophore labeled oligonucleotides were used in PCR nucleic acid amplification reaction.

Use of both oligonucleotide primers of amplification reaction as labeled oligonucleotide primers does not affect PCR amplification reaction much :-

FAM labeled forward primer (seq.no.19) and JOE labeled reverse primer (seq no.20) as well as same forward and reverse primers as unlabeled oligonucleotide primers (10 & 13) were used in amplification at approximately 0.4μM (micro molar) concentration. The sizes of the amplification products were same and the yield of the amplification product of labeled primers was slightly less in comparison to the yield of the amplification product of unlabeled primers (Fig Nos.32 & 33).

FAM labeled forward primer (seq No.21) and JOE labeled reverse primer (seq No.20) as well as same forward and reverse primers as unlabeled oligonucleotide primers (seq.nos. 10 & 13) were used in amplification reactions. The sizes of the amplification products were same and the yield of the amplification product of labeled primers was slightly less in comparison to the yield of the amplification product of unlabeled primers (seq.nos.10 and 13). However, there was a primer dimer formation in this case of seq.nos.20 & 21 and no primer dimer formation in the previous case (seq.nos. 19 and 20). This primer dimer may be the homodimer of the FAM labeled forward primer (seq.no.21). This demonstrate that oligonucleotide primer labeled internally at the extreme 3' end base can be extended by Taq DNA polymerase more or less with same efficiency as that with unlabeled oligonucleotide primer. This also demonstrate that internal fluorophore labeling near 3' ends of both the forward and reverse primer help in eliminating primer dimer formation due to lowering of melting temperature of both primers.

In all three cases the yield of the amplification product utilizing fluorophore labeled primers was compared with the yield of the amplification product of the

size close to that of primer dimer using unlabeled primers. The yield of amplification product of the size close to that of primer dimer is many fold higher in comparison to that of larger size amplification product. And also it is known that efficiency of utilization of flurophore labeled nucleotide by DNA polymerase depend on the linker length between the nucleotide and the fluorophore (Nucl. Acid Research 1994, 22 (16), 3418-3422). The yield of the amplification product of fluorophore labeled primers can be further improved by varying length of the linker linking the fluorophore to the oligonucleotide /oligonucleotides.

Ex 3 - Detection of an amplification product by fluorescence energy transfer (FRET) between donor flurophore FAM and acceptor flurophore JOE on two amplification primers of an amplification reaction.

To detect amplification product FAM labeled forward primer (Seq.No.19) and JOE labeled reverse primer (Seq.No.20) were used in PCR amplification reaction with and without template DNA. After amplification reaction, amplification reaction mixtures were illuminated with FAM specific excitation light of 488 nm and characteristic emissions at 550 nm of JOE as well as emission of FAM 520nm were measured. There was sizeable increase in JOE emission and decreased FAM emission from the reaction mixture containing template DNA, whereas there was almost negligible increase in JOE emission from the reaction mixture containing no template DNA (Fig. 34).
When Rhodamine was used in place of JOE of reverse primer keeping the primer sequence and labeling position unchanged there was 50% reduction in FAM emission at the end of the amplification.

Ex 4 Use of hair-pin quenched oligonucleotide reverse primer labeled with an acceptor fluorophore FAM near 3' end and a quencher DABCYL at 5' end and a donor fluorophore FAM labeled forward primer resulted in higher FRET signal to noise ratio :-

Amplification reaction was carried out with a donor fluorophore FAM labeled forward primer labeled internally near 3' end (seq no 19) and a hair-pin reverse primer labeled internally with an acceptor fluorophore FAM near 3' end and a

quencher DABCYL at 5' end (seq no 23) with and without template DNA (Leishmania donovani chromosomal DNA). After the amplification reaction the reaction mixtures were illuminated with FAM specific excitation light of 488nm wavelength and characteristic emissions of FAM at 530nm was measured. There was large increase in FAM emission from the reaction mixture containing the template DNA whereas there was almost no increase in FAM emission from the reaction mixture containing no template DNA (Fig 35) A signal to noise ratio of approximately 60 was observed. However, we feel that FAM and DABCYL labeled hair-pin reverse primer contained free FAM labeled primer and the FAM labeling was not very good. Signal to noise ratio can be improved further by improving the labeling and purification of the labeled primers particularly the fluorophore and quencher labeled primer. Use of a suitable spacer for attaching the fluorophore to the oligonucleotide may result in still higher signal to noise ratio.

Ex-5 Reduction of noise from primer dimer formation in FRET based detection or quantitation of amplification product or reaction :-

For reduction of noise from primer dimer the reverse hair-pin primer (seq.no.23) was labeled near its 3' end with a donor fluorophore FAM and at 5' end with quencher DABCYL. The forward primer (seq.no.24) was labeled near its 3' end with the quencher DABCYL. The forward and the reverse primers were designed to amplify a 64bp segment of Leishmania donovani gp 63 gene and the two primers were so designed that when incorporated into the amplification product the FAM of the reverse primer and the quencher DABCYL on both forward as well as reverse primer remained more than 15 base away from FAM on either side thus allowing FAM incorporated into the amplification product to emit its own characteristic emission, which could be measured. In case of primer dimer formation the quencher DABCYL near 3' of the forward primer would come proximal (within FRET distance) to the fluorophore FAM resulting in quenching of FAM emission, thus reducing or nullifying contribution of primer dimer towards fluroescence emission from FAM due to separation of FAM and DABCYL of forward primer. A signal to noise ratio of 50 was observed (fig 36). Signal to noise ratio was much less than expected higher value due to presence of free FAM

labeled primer in the FAM-DABCYL double labeled primer. Signal to noise ratio can be improved further by improving the primer labeling and purification method.

Ex-6 Close tube format

In this Leishmania donovani chromosomal segment was amplified for 30 cycles using FAM labeled forward primer (Seq.No.19) and FAM and quencher DABCYL labeled hair-pin quenched reverse primer (Seq.No.23) in presence of 100 ngs. of Leishmania donovani chromosomal DNA and primers at a conc. of 175pM. As a control same amplification reaction was carried out in absence of template DNA. The primers were designed such that fluorescence resonance energy transfer signal is generated only when the amplification product is formed, i.e. the forward and reverse primers get incorporated into the two opposite strand of the amplification product in right proximity. A signal to noise ratio of more than sixty was observed (fig No.35). This method eliminates the carry-over contamination problem associated with PCR, simplifies the process and allow real time qualification of target nucleic acid sequence.

Materials and method:

Following oligodeoxynucleotides complementary to a 60 bp segment of a 70 base pair synthetic target sequence were chemically synthesized on an Applied Biosystem oligosysnthesizer.

Sequence No.1 - 5'-ACT TAA GTT AGA GCG TTT GC-3' as forward primer complimentary to the 60 bp segment of synthetic target sequence

Sequence No.2- 5'-TGG TAG TAT GTG ATT TAG TC-3' as a reverse primer complementary to the 60 bp segment of synthetic target sequence.

Sequence No.3 -5'-TAC ACT TAA GTT AGA GCG TTT GCG CCC ACT ACG ACG GTT G-3' for the preparation of 70 bp synthetic target sequence.

Sequence No.4 -5'-G TTT TTG TGG TAG TAT GTG ATT TAG TCA TTC AAC CGT CGT AGT G -3' for the preparation of 70 bp synthetic target sequence.

Fluorescent dye labeled primers synthesized for the detection of 60bp synthetic target sequence are shown below with the position of label.

Sequence No.5-5'- ACT TAA GTT AGA GCG TTT GC-3'

FAM

FAM labeled forward primer for 60bp synthetic DNA template. FAM is on T at base position 18.

Different 3'end JOE labeled oligonucleotides were used in combination to the above FAM labeled primer to find the FRET distance between FAM and JOE.

Following oligodeoxuynucleotide primers complementary to a segment of Leishmania donovani chromosomal target gp63 gene (Accession No.M60048) were synthesized.

Sequence No.6- 5'-ACG GAG CGG CTG AAG GTG C-3' as a forward primer to amplify 40 base pair segment (base positions 566 to 605) of gp63 gene of Leishmania donovani.

Sequence No.7- 5' -AGG TGC ATC CAC TTG TCC TGC ACC TGC-3' as a reverse primer to amplify 40 base pair segment (base positions 566 to 605) of gp63 gene of Leishmania donovani.

Sequence No.8- 5' -AGG CAG ATG GCG CCT GCC TCG-3' as a forward primer to amplify 36 base pair segment (base positions 1094 to 1129) of gp63 gene of Leishmania donovani.

Sequence No.9- 5'- ATC CGG CGC TGT AGT ACC CCG CAT C-3' as a reverse primer to amplify 36 base pair segment (base positions 1094 to 1129) of gp63 gene of Leishmania donovani.

Sequence No.10- 5' -GGG GTA CTA CAG CGC CCT GA-3' as a forward primer to amplify 40 base pair segment (base positions 1114 to 1153) of gp63 gene of Leishmania donovani.

Sequence No.11 -5'-ATG GCC ATG TCC TGG AAG ATG GCC ATG G-3' as a hair-pin reverse primer with eight base stem structure to amplify 40 base pair segment (base positions 1114 to 1153) of gp63 gene of Leishmania donovani

Sequence No.12 - 5'-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG-3' as a hair-pin reverse primer with nine base stem structure to amplify 40 base pair segment (base positions 1114 to 1153) of gp63 gene of Leishmania donovani

Sequence No.13 - 5' - GTC CTG GAA GAT GGC CAT GG-3' as a linear reverse primer to amplify 40 base pair segment (base positions 1114 to 1153) of gp63 gene of Leishmania donovani

Sequence No.14 - 5' -CTG CAC ACG GAG CGG CTG AA-3' as a forward primer to amplify 544 base pair segment (base positions 560 to 1103) of gp63 gene of Leishmania donovani.

Sequence No.15 –5'–GGA CGA GCT CAT GGC GCC TG-3' as a reverse primer to amplify 544 base pair segment (base positions 560 to 1103) of gp63 gene of Leishmania donovani

Sequence No.16 - 5'- GTC CTG TTC ACC TTC CAC TG-3'as a reverse primer to amplify 47 base pair segment (base positions 566 to 612) of gp63 gene of Leishmania donovani

Sequence No.17 - 5' -GCT CAT GGC GCC TGC CTC G -3' as a forward primer to amplify 39 base pair segment (base positions 1090 -1128) of gp63 gene of Leishmania donovani.

Sequent No.18-5' -GCG TGT AGT ACC CCG CAT C-3' as a reverse primer to amplify 39 base pair segment (base positions 1090-1128) of gp 63 gene of Leishmania donovani

Following fluorescent labeled oligonucleotide primers were synthesized for the detection of a 40bp segment of gp63 gene of Leishmania donovani

Sequence No.19 - 5' - GGG GTA CTA CAG CGC CCT GA-3'

|

FAM

20 base FAM labeled forward primer for amplification of 40bp segment (base position 1114 to 1153) of gp63 gene of Leishmania donovani. FAM is on T at base position 18.

Sequence No.20-5' -GTC CTG GAA GAT GGC CAT GG-3'

|

JOE

JOE labeled linear reverse primer for amplification of 40bp segment (base positions 1114 to 1153) of gp63 gene of Leishmania donovani. JOE is on T at base position 18.

Sequence No.21 -5' - GGG GTA CTA CAG CGC CCT-3'

|

FAM

18 base FAM labeled forward primer for amplification of 40 base pair segment (base position 1114-1153) of gp63 gene Leishmania donovani. FAM is on T at base position 18.

Sequence No.22 –

5' -DABCYL -ATG GCC ATC GTC CTG GAA GAT GGC CAT GG-3'

|

JOE

JOE and DABCYL dual labeled quenched hair-pin reverse primer with nine base stem structure to amplify 40 base pair segment (base positions 1114 to 11153) of gp63 gene of Leishmania donovani. JOE is on base T at base position 27 and DABCYL at 5' end.

Sequence No.23 –

5'- DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG-3'

|

FAM

FAM and DABCYL dual labeled quenched hair-pin reverse primer with 9 base stem structure to amplify 64 base pair segment (base positions 1090 – 1154) of gp63 gene of Leishmania donovani. FAM is on base at base position 27 and DABCYL at 5' end.

Sequence No.24 - 5' -GCT CAT GGC GCC TGC CTC G-3'

|

DABCYL

DABCYL labeled forward primer for amplification of 64 base pair segment (base position 1090-1153) of gp63 gene of Leishmania donovani. DABCYL is on base T at base position 17.

Description of the figures :

Fig 1Aand 1B The structure of the hair-pin oligonucleotides of the invention in closed quenched (a) and open signal emitting (b) states ; open circle (F) being the donor or acceptor fluorophore and the solid-circle being the quencher.

Fig.2 Schematic illustration of the use of donor fluorophore labeled linear forward primer and acceptor labeled linear reverse primer in the detection and/or quantitation of an amplification product produced from PCR amplification. A fluorescence energy transfer signal is generated only when the fluorophore labeled primers get incorporated into the two strands of the double stranded amplification product, (D) donor fluorophore (A) acceptor fluorophore.

Fig.3 Schematic illustration of the use of donor and acceptor labeled quenched hair – pin primers in PCR amplification, (A) is acceptor, (D) is donor, (Q) is quencher.

Fig.4 Schematic illustration of linear donor labeled forward primer and acceptor labeled quenched hair – pin primer in PCR amplification (A) acceptor fluorophore, (D) is donor, (Q) is quencher.

Fig5 Schematic illustration of the use of unlabeled reverse primer, acceptor labeled quenched hair – pin forward primer and donor labeled quenched hair – pin probe in PCR amplification.

Fig6 Schematic illustration of the use of donor fluorophore labeled linear forward primer, acceptor fluorophore labeled hair-pin quenched reverse primer and blocker in triamplification. A fluorescence resonance energy transfer signal is generated only when the donor fluorophore labeled forward primer and acceptor fluorophore labeled reverse primer gets incorporated into the two strands of the amplified product; (D) donor fluorophore (A) acceptor fluorophore and (Q) quencher.

Fig.7 Schematic illustration of the use of acceptor fluorophore labeled hair-pin quenched forward and donor fluorophore labeled reverse primer in nucleic acids

sequence based amplification (NASBA). A fluorescence resonance energy signal is generated only when the acceptor labeled forward primer and donor labeled reverse primer get incorporated into the two strands of the amplification product (D) donor (A) acceptor (Q) quencher.

Fig.8 Sequence of 70 bp synthetic template DNA

Fig 9 Sequence of the 40 bp segment (base position 1114-1153) of the Leishmania donovani gp 63 gene (Gene Accession No.M60048).

Fig.10 Sequence of 40 base pair segment (base position 566-605) of the Leishmania donovani gp 63 gene (Gene Accession No.M60048)

Fig.11 sequence of 36 base pair segment (base position 1094-1129) of the Leishmania donovani gp63 gene(Gene Accession No.M60048)

Fig.12 FAM labeled oligonucleotide primer for amplification of 70bp synthetic template.

Fig.13 FAM labeled forward primer (seq.no.19) for amplification of 40bp segment (base position 1114-1153) of Leishmania donovani gp63 gene.

Fig.14. JOE labeled reverse primer (seq.no.20) for amplification of 40 bp segment (base position 1114-1153) of Leishmania donovani gp63 gene.

Fig.15. FAM labeled forward primer (seq.no.21) for amplification of 40 bp segment (base position 1114-1153) of gp63 gene of Leishmania donovani.

Fig.16 JOE and DABCYL labeled reverse primer seq.no.22 for amplification of 40bp segment (base position 1114-1153) of Leishmania donovani gp63 gene.

Fig.17 FAM and DABCYL labeled reverse primer (seq.no.23)

Fig.18 DABCYL labeled forward primer (seq.no.24)

Fig.19 sequence of 610 base pair segment (base position 560-1170) of the Leishmani donovani gp63 (Gene Accession No.M60048)

Fig.20 Gel image illustrates that amplification of specific amplification product and no primer dimer is formed using primer pairs seq.nos.8 and 13; 14 and 15 and 10 and 13. From left to right lane no.1 and 2 amplification product (60 base pair) of primer pair seq.nos.8 and 13 in duplicate, lane no.3 and 4 amplification product (544 bp) of primer pair seq.nos.14 and 15 in duplicate, and lane no.5 and 6 amplification product (40 bp) of primer pair seq.nos.10 and 13 in duplicate.

Fig.21 Gel image illustrates use of an amplification product of the size close to that of primer dimer for nucleic acid amplification helps in eliminating or reducing non-specific amplification product formation. Upper gel image is image of the gel at lower sensitivity while the lower gel image is the same image at higher sensitivity of the phosphor imager. From left to right lane no.1 and 2 amplification product of the primer pair seq.no.6 and 7 in duplicate, lane no.3 and 4 amplification product of the primer pair seq.no.8 and 9 in duplicate lane no.5 and 6 amplification product of the primer pair seq.no.10 and 13 in duplicate, lane no.7 and 8 amplification product of the primer pair seq.no.8 and 13 in duplicate, lane no.9 and 10 amplification product of the primer pair seq.no.14 and 15 in duplicate.

Fig 21A- Gel image also illustrates use of an amplification product of the size close to that of primer dimer for nucleic acid amplification helps in eliminating or reducing non-specific amplification product formation. From left to right lane no.1 amplification product of the primer pair seq.no.6 & 16 in absence of the template DNA, lane no. 2 amplification product of the primer pair seq.no.6 & 16 in presence of the template DNA, lane no.3 and 4 amplification products of the primer pair seq.no. 13 and 17 in absence and in presence of template DNA respectively, lane no.5 amplification product of the primer pair seq.no.10 and 13 in absence of template DNA, lane no.6 and 7 amplification product of the primer pair seq.no.10 and 13 in presence of template DNA in duplicate, lane no.8 and 9

amplification product of the primer pair seq.no.17 and 18 in absence and in presence of template DNA respectively.

Fig.22 gel image illustrates use of an amplification product of the size close to that of primer dimer for nucleic acid amplification helps in eliminating or reducing non-specific amplification product formation. Upper gel image is image of the gel at lower sensitivity while the lower gel image is the same image at higher sensitivity of the phosphor imager. From left to right lane no.1 and 2 amplification product of the primer pair seq.no.14 and 15 in duplicate, lane no.3 and 4 amplification product of the primer pair seq.no.6 and 15 in duplicate, lane no.5 and 6 amplification product of the primer pair seq.no.10 and 13 in duplicate, lane no.7 and 8 amplification product of the primer pair seq.no.14 and 9 in duplicate, lane no.9 and 10 amplification product of the primer pair seq.no.14 and 13 in duplicate.

Fig.23 Gel image illustrates use of amplification product of the size close to that of primer dimer for nucleic acid target amplification result in higher amount of amplification product. The amplification product of primer pair 14 and 15 in presence of 50ng of L.donovani DNA was analyzed by 10 per cent PAGE. Gel image from left to right lane no.1 and 2 amplification product formation after 10 cycles, lane no.3 and 4 same after 15 cycles, lane no.5 and 6 same after 20 cycles, lane no.7 and 8 same after 25 cycles, lane no.9 and 10 same after 30 cycles. The product formed in the beginning, i.e., in the early cycles is non – specific product of size close to 100 base pair. The specific product, a high molecular weight product, is formed in later cycles and is the top most but one band.

Fig.24 Gel image illustrates use of amplification product of the size close to that of primer dimer for nucleic acid target amplification result in higher amount of amplification product. The amplification product of primer pair 10 and 13 in presence of 50ng of L.donovani DNA Gel image was analyzed by 15 per cent PAGE. From left to right lane no.1 and 2 amplification product formation after 10 cycles, lane no.3 and 4 same after 15 cycles, lane no.5 and 6 same after 20

cycles, lane no.7 and 8 same after 25 cycles, lane no.9 and 10 same after 30 cycles.

Fig.25 Gel image use of an amplification product of the size close to that of primer dimer for nucleic acid amplification based analysis can result in higher throughput. From left to right lane-1, DNA marker for primer dimer formation between the primers seq.6 and seq.no.16. Lane-2, primer dimer formation by the primers seq.no.6 and seq.16 in absence of template DNA lane-3, amplification product formation by the primer seq.no.6 and seq.no.16 in presence of Leishmania donovani chromosomal DNA (100 ng). Lane-4, primer dimer formation between the primers seq.no.17and seq.no.13 in absence of template DNA Lane-5, amplification product formation by the primers seq.no.17 and seq.13 in presence of Leishmania donovani DNA. Lane-6, primer dimer formation between the primers seq.no.10 and seq.no.13. Lane-7, amplification product formation by the primers seq.nos.10 and 13 in presence of L.donovani DNA. Lanes 9 & 10 primer dimer formation and 100bp amplification product formation by primers designed from □□DNA. A denaturation time of 10 secs and temp 95°C and annealing time of 2 secs and annealing temperature of 60°C were employed for 30 cycles and was analyzed on 15 percent PAGE.

Fig.26. The gel (20 per cent PAGE) well no.1 primer seq.nos.14 and 15, 0.35μM each, L.donavani chromosomal template DNA 100ng. Well No.3 44 bp DNA marker, well No.5 and 6 primer seq.nos.10 and 11, 0.18μM each and L.donavani chromosomal template DNA 100ng. Well no.8 and 9 primer seq.no.10 and 11 0.18μM each and no L.donovani chromosomal template DNA Lower bands are small non-template dependant primer extension products.

Doublet products may either be two amplification products one being single A moieties added at 3' ends or most probably due to formation of smaller product from the designed amplification product reaching higher concentration due to the presence of six nucleotide palindromic sequence at 3' end of the primer seq 13. Primer seq 13 inspite of having six nucleotide complementation did not form primer dimer in absence of template DNA most probably due to approximately

50% GC content and not more than two GC or combination thereof among the above six nucleotides.

Fig.27. The gel (20 per cent PAGE) well no.2 primer seq.nos.14 and 15 0.35μM each, L.donovani chromosomal template DNA 100ng. Well no.4 and 5 primer seq.nos.10 and 13, 0.18μM each and L.donovani template DNA 100ng. Well no.7 and 8 primer seq.no.10 & 13, 0.18μM each and no template DNA well 10, 44bp DNA marker.

Fig.28. Left gel (20 per cent PAGE) well no.1 primer seq.nos.14 and 15, 0.35μM each and L.donovani DNA 100ng. Well 3 and 4 primer seq.nos. 10 and 12, 0.18μM each and L.donovani DNA well 6 and 7 primer seq.nos.10 and 12, 0.18μM each and no template DNA well no.9-44 bp DNA marker. Right gel (20 per cent PAGE) well no.1 - primer seq nos.14 and 15, 0.35μM each and L.donovani template DNA 100ng. Well nos.3 and 4 primer seq.nos. 10 and 12, 0.35μM each and L.donovani template DNA 100ng. Well nos. 6 and 7 - primer seq.nos. 10 and 12, 0.35μM each and no template DNA. Well no.9-44 bp DNA marker.

Doublet products may either be two amplification products one being single A moieties added at 3' ends or most probably due to formation of smaller product from the designed amplification product reaching higher concentration due to the presence of six nucleotide palindromic sequence at 3' end of the primer seq 13. Primer seq 13 inspite of having six nucleotide complementation did not form primer dimer in absence of template DNA most probably due to approximately 50% GC content and not more than two GC or combination thereof among the above six nucleotides.

Fig.29 Upper left gel well no.1 and 2 primer seq.nos. 10 and 12, 0.35μM each and 100ng. L.donovani chromosomal DNA. Well no 4 and 5 primer seq nos.10 and 11, 0.18μM each and 100ng L.chromosomal DNA. Well 7 and 8 primer seq nos.10 and 11, 0.35μM each and 100ng L.chromosomal DNA. Well 10-44 bp DNA marker (20 per cent PAGE). Upper right gel well no.1 and 2 - primer seq

nos 10 and 13 0.4μM each and no template DNA. Well nos.4 and 5 - primer seq nos.10 and 12, 0.35μM each and no template DNA. Well nos. 7 and 8 - primer seq nos.10 and 11, 0.18μM each and no template DNA. Well no.10 - 44bp DNA marker (20 per cent PAGE). Lower gel well 1 and 2 - primer seq nos 14 and 15, 0.35μM and 100ng L.donovani DNA. Well 4 and 5 - primer seq nos 10 and 13, 0.35μM each and 100ng L.donovani DNA. Well no.7 – 44bp DNA marker (20 per cent gel). In the gels of the fig. 29, ^{32}P labeled dATP has not got out of the gel.

Fig.30. 15 per cent denaturing PAGE well no 2 and 3, 5' end labeled primer seq no.11 and cold primer seq.no.10, 0.18μM each plus L.donovani chromosomal template DNA. Well no.5-5' end label primer seq no.11 well no.6-5' end labeled primer seq no.,10 well no 7-44 bp marker DNA well no.9 and 10-5' end labeled primer seq.no.10 and cold primer seq no.11 each 0.18μM plus L.donovani chromosomal template DNA. Well no.12 & 13-5' end labeled primer seq.no.11 and cold primer seq.no.10 each 0.18 μM and no template DNA. Well no.15 - 44bp DNA MARKER. Well no 17 and 18-5' end labeled primer seq 10 and cold primer seq no.11 each 0.18μM and no template DNA. 5' ends were labeled with ^{32}P.

Fig.31 15 per cent denaturing PAGE well No.1 and 2-5' end labeled primer seq no.11 and unlabeled primer seq no.10 each 0.18μM without template DNA. Well no.4-44bp DNA marker. Well no.5-5' end labeled primer seq no.10. Well no.6-5' end labeled primer seq no.11. Well no.8 and 9-5' end labeled primer seq.no.11 and unlabeled primer seq.no.10 each 0.18μM and L.donovani template DNA 100ng. 5' ends were labeled with ^{32}P.

Fig.32 & 33 Illustrates that use of both oligonucelotide primers of amplification reaction as fluorophore labeled oligonucleotide primers (labeled near 3' end) does not affect PCR amplification reaction.

Fig 32 Ethidium Bromide stained gel picture, Lane 1 & 3 Primer seq nos 19 & 20 0.35□M each, 100ng L.donovani DNA, Lane 5 Primer seq nos 10 and 13 each

0.18□M, 100ng L.donovani DNA, Lane 7 and 9 Primer sequence nos.20 and 21 0.35□M each, 100ng L.donovani DNA.

Fig 33 is same as fig 32 but in this case [□^{32}P] dATP was used for labeling the products.

Fig.34 Detection of an amplification product as by fluorescence resonance energy transfer (FRET) between FAM and JOE on two oligonucleotide primers.

Fig.35. Illustrates use of hair-pin quenched oligonucleotide reverse primer labeled with an acceptor fluorphore FAM near 3'end and a quencher DABCYL at 5'end and a donor fluorophore FAM labeled forward primer resulted in higher FRET signal to noise ratio.

Fig.36. Illustrates reduction of noise from primer dimer formation in FRET based detection or quantitation of nucleic acid target sequence or amplification product in amplification reaction.

General Methods:

1. All oligodeoxynucleotides were synthesized chemically by standard solid phase phosphotriester chemistry.

2. Single fluorophore labeling of the oligodeoxynucleotide primers.

The single labeling of the oligonucleotide primers with fluorophore at or near 3' end was done through incorporation of a primary amino group by incorporating amino modified T – base (amino modified C_6dT) during synthesis as described by Ju et al (Proc. Natl. Acad. Sci. USA, 1995, 92, 9347 – 9351) and subsequent incorporation of fluorescent dyes into designated position of the oligonucleotides. Syntheized oligonucleotides were desalted and FAM (as donor) and JOE (as an acceptor) were attached to a modified thymidine residue of the forward and reverse primers. Labeled oligonucleotides were purified by HPLC. Internal single fluorophore labeled oligonucleotides are also available commercially.

3. Fluorophore and quencher double labeling of hair-pin oligodeoxy nucleotide primers:

The labeling of the hair-pin oligodeoxynucleotide primers with fluorophore near 3' end and quencher at 5' end were done through incorporation of a primary amino group by incorporating amino modified T base (amino modified C_6dT) during synthesis as described by Ju et al (Proc. Natl. Acad. Sci. USA, 1995, 92, 9347-9351) and incorporation of a thiol group at the 5' end during synthesis using thiol phosphoramidite. After desalting the oligodeoxynucleotides are reacted to N-hydroxy succinamide derivative of the fluorophore, purified by HPLC and are subsequently reacted to N- (2 iodoethyl) trifluoroacetamide, desalted and reacted to DABCYL N-hydroxy succinamide (similar to fluorophore labeling of the above oligonucleotide; PNAS 1995, 92, 9347- 9351).

Alternatively the aminoderivative of DABCYL prepared by reacting DABCYL-N-hydroxy succinamide to ethylenediamine and purified by HPLC (Bioconjugate chem. 2000, 11,161-166) could be attached to the 5' thiol group of the oligonucleotide through iodo/bromo acetic acid or iodoacetic acid N-hydroxysuccinamide linker (Nucl acids Res. 2001, 29, 955-959) or through a N-succinimidyl (4-iodoacetyl) aminobenzoate linker (Anal chem.. 1997, 69, 2438-2443).

4. HPLC purification of oligonucleotide:

5' DABCYL and 3' fluorophore labeled oligonucleotides were purified by HPLC on C – 18 reverse phase column using linear gradient of 0.1M triethyl ammonium acetate pH 6.5 and 0.1M triethylammonium acetate in 75% acetonitrile pH 6.5.

5. Measurement of Energy transfer or FRET :-

Fluorescent resonance energy transfer (FRET) measurements were made in a Hitachi F4010 fluorescence spectrophotometer. Excitation wavelength was 488 nm and the emission spectra and measurements were taken between 500 nm and 600 nm.

6. Preparation of *Leishmania donovani* chromosomal DNA:

Leishmania donovani carrying cells were washed in PBS twice and pelleted at 3K, 10 min at 24^{0}C. Cells were then resuspended in appropriate volume of Lysis buffer (150mM NaCl, 10mM EDTA, 10mM Tris – HCl pH 7.5, 40□l of 10% SDS per ml of buffer, 200□g/ml Proteinase K) in a 15ml Falcon tube. The tube was vortexed hard and incubated at 37^{0}C overnight or until the cell pellet dissolved. Phenol extraction was carried out with equal volume Tris equilibrated Phenol. The resulting suspension was centrifuged in microfuge at 3K for 10 min at RT.

7. PCR conditions:

A. PCR conditions for synthetic target sequence amplification:

Amplification of the synthetic 60 bp target was performed in 100□l volume of 20mM Tris – HCl (pH – 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200□M each dNTP, 400 - 500nM each of the upstream primers, 0.01% gelatin, 3.0 units of Taq DNA polymerase, 1-5ng of synthetic target sequence and thermal cycling of 2 minutes initial denaturation followed by 30 secs. Denaturation at 94^{0}C, 1 min annealing at 50^{0}C and 30 secs. extension at 72^{0}C, 30 cycles and final extension at 72^{0}C for 2 mins.

B. PCR condition for amplification of *Leishmania donovani* gp63 target sequence:

Amplification of gp63 target sequence of *Leishmania donovani* was performed in 100□l volume of 20 mM Tris HCl pH-8.3, 50mM KCl, 1.5mM $MgCl_2$, 200□M each dNTP, 200 -400nM each of the upstream and downstream primers, 0.01% gelatin, 3.0 units of Taq DNA polymerase, 100ng of chromosomal DNA and thermal cycling of 4 mins initial denaturation, followed by 30 seconds denaturation at 94^{0}C, 1 min annealing at 60^{0}C and 30 seconds extension at 72^{0}C, 30 cycles and final extension at 72^{0}C for 7 mins.

The PCR product formation was checked in 4% agarose gel run in 1XTAE buffer and the PCR product was quantitated by using ^{32}P or ^{33}P labeled dNTP and separating the labeled amplification product from unincorporated dNTPs by PEI cellulose TLC in 1M Na-phosphate pH 3.5 buffer or 10-20% non-denaturing polyacrylamide gel electrophoresis and gels were scanned in a Fuji Model Fuji film BAS-1800 Phosphorimager.

C. PCR condition for amplification of *Leishmania donovani* gp63 target sequence (using
Fluorophore labeled primers):

Amplification of gp63 target sequence of *Leishmania donavani* was performed in 100□l volume of 20 mM Tris HCl pH-8.3, 50mM KCl, 1.5mM $MgCl_2$, 200□M each dNTP, 200 -400nM each of the upstream and downstream primers, 0.01% gelatin, 3.0 units of Taq DNA polymerase, 100ng of chromosomal DNA and thermal cycling of 4 mins initial denaturation, followed by 30 seconds denaturation at 94^{0}C, 1 min annealing at 55^{0}C and 30 seconds extension at 72^{0}C, 30 cycles and final extension at 72^{0}C for 7 mins.

The PCR product formation was checked in 4% agarose gel run in 1XTAE buffer and the PCR product was quantitated by using ^{32}P or ^{33}P labeled dNTP and separating the labeled amplification product from unincorporated dNTPs by PEI cellulose TLC in 1M Na-phosphate pH 3.5 buffer or 20% non-denaturing polyacrylamide gel electrophoresis and gels were scanned in a Fuji phosphor imager Model No. Fuji film BAS-1800.

8. Fluorescence measurement

A Hitachi F 4010 Fluorescence spectrophotometer was used to measure the fluorescence spectra and fluorescence of the individual samples. 20□l reaction mixture was diluted to 1000 □l with water and placed into a 1.0 ml cuvette at a temperature of 37 to 40^{0}C. For the FAM/JOE FRET pair 488nm excitation wavelength was used for excitation of FAM and the spectrum of JOE was measured between 500 and 600nm.

9. Spectroscopic properties and sequences of linear oligonucleotides and estimation of FRET distance:

The sequence of the oligonucleotides and the location of the fluorescent labels on them are given in 'Materials and Methods'. The donor FAM labeled forward primer Seq. no. 3 consists of 20 nucleotides and carries the FAM label at base position 18. The reverse primer Seq. no. 2 consists of 20 nucleotides. Acceptor JOE labeled oligonucleotide probes were labeled at their 3' end with the fluorophore JOE.

10. Monitoring of PCR by sensitized emission and estimation of optimum FRET distance:

To demonstrate that sensitized emission could be used to monitor PCR amplification reaction or amplification product, the synthetic template (Sequence given in Fig. 8) was amplified using the FAM labeled forward primer (Sequence no. 5) and the reverse primer (Sequence no 2) in the presence of 3' JOE labeled probes which bring the JOE label at distances of 5, 10, 15, 20 bases from FAM label in amplified product. After PCR amplification the tube was denatured and annealed once more and the amplified product was measured by illuminating the reaction mixture with FAM excitation wavelength of 488nm light and measuring the emission of JOE at 553nm at 37 – 40^{0}C. There was a decrease in FAM emission (i.e., quenching of donor fluorescence) and increase in JOE emission. Energy transfer was observed upto the distance of 20 base pair and maximum energy transfer was observed at a distance of 5 base pair. The JOE labeled probes are not shown.

11.Design of fluorophore labeled oligonucleotide primers:

General consideration of the primer design is same as that of Ex.1E. Further a primer pair of high stringency can be labeled at or a few nucleotides away

(preferably 2 – 4) from the 3' ends while primer pair of low stringency should be labeled at a few nucleotides away (preferably 2 – 4) from 3' ends.

WE CLAIM :

1. A method of detection of a target nucleic acid sequence by nucleic acid amplification reaction, comprising use of two oligonucleotides as a pair of primers for amplification of said target nucleic acid sequence, with one primer being labeled with a donor MET moiety, and the other primer being labeled with an acceptor MET moiety, the said donor and acceptor MET moieties belonging to a molecular energy transfer pair and so configured that the donor and the acceptor moieties come within MET distance, 10 – 80 Angstrom or the nucleotides to which the MET moieties are attached are separated by 2 – 20 or 25 nucleotides.

2. A method of claims 1, wherein the labeled oligonucleotide primers are labeled at or near their 3' ends , preferably near 3' end,with donor MET moiety or acceptor MET moiety.

3. The method as claimed in any one of claims 1 & 2, wherein the oligonucleotide labeled with the acceptor moiety is provided in quenched condition with a quencher following conventional methods such that the quencher is capable of absorbing the emission energy of the acceptor and quenching the same.

4. The method as claimed in any one of claims 1 to 3 wherein both the acceptor and the donor-MET moieties are provided quenched with individual quenchers.

5. A method of claim 1to 4 wherein a first oligonucleotide primer pair selected to amplify the target nucleic acid at appropriate concentrations, a second oligonucleotide primer pair selected to amplify a second segment of the first segment at appropriate concentration is used in nested PCR and the said second oligonucleotide primer pair is any of the labeled oligonucleotide primer pairs of the claims 1- 4.

6. A method of claim 1 to 5 wherein a first oligonucleotide primer pair selected to amplify a first segment of the target nucleic acid is used at appropriate concentration, where one of the said oligonucleotide primer pair is a first member of the labeled oligonucleotide primer pairs of claims 1- 4. A third oligonucleotide suitably labeled for MET and designed to amplify a second segment of the first segment in association with the above first member is nested.

7. A method of detection of target nucleic acid sequence by nucleic acid amplification reaction, comprising use of two oligonucleotides 1&2 as a pair of primers for amplification of said target nucleic acid sequence, with one of them being labeled with a donor /acceptor MET moiety, and an oligonucleotide 3 labeled with a complementary acceptor/donor MET moiety of a molecular energy transfer pair, where oligonucleotide 3 is complementary to the target sequence and is not extended by the polymerase, both the labeled oligonucleotides being labeled suitably at or near their 3' ends.

8. A method as claimed in claim 1-7, wherein said nucleic acid amplification reaction comprise any known nucleic acid amplification reaction comprising polymerase chain reaction comprising the steps of adding a polymerase, reaction buffer, deoxy nucleoside triphosphates in addition to the effective amounts of amplification primers to the sample, cycling the sample, between at least a denaturation temperature and an annealing temperature, exciting the reaction mixture with the donor exciting radiation or light, measuring the emission of the acceptor MET moiety, optionally that of the donor.

9. A method of detection of target nucleic acid sequence by nucleic acid amplification reaction comprising (i) use of two oligonucleotides as a pair of primers for amplification of said target sequence ; (ii) the 3' ends of said pair of primers being separated from one another by 0-25 nucleotide pairs in the final amplification product ; and (iii) employment of time periods for

denaturation, less than 10 seconds, annealing of less than 5 seconds and extension of 0 second in each cycle.

10. The method of claim 9 with a time period less than 15 seconds for annealing.

11. The method as claimed in anyone of claims 1 – 4 and 9 – 10 used for high through put PCR or nucleic acid target analysis.

12. The method as claimed in any one of claims of 9 - 11 where in denaturation time of less than 1 minute preferably less than 20 seconds and annealing time of less than 30 seconds, preferably less than 15 seconds are employed.

13. The method as claimed in any one of claims of 1 to 6 and 8 to 12 wherein an amplification product of the size of 20 to 65 nucleotide pairs preferably 30 to 65 nucleotide pairs is generated for nucleic acid target analysis.

14. The method as claimed in any one of claims 1 to 8 comprising additionally one more oligonucleotide labeled at or near its 5 end with a quencher complementary to the acceptor labeled oligonucleotide.

15. The method as claimed in any one of claims 1 to 8 comprising additionally two more oligonucleotides and labeled at or near their 5' ends with quenchers to the donor and acceptor MET moieties complimentary to the donor and acceptor labeled oligonucleotides respectively.

16. The method as claimed in any one of claims 1 to 8 wherein the acceptor labeled oligonucleotide is linked with an organic linker to another suitable oligonucleotide complementary to this acceptor labeled oligonucleotide and labeled with a quencher at or near its 5'.end.

17. The method as claimed in any one of claims1 to 8 wherein both the acceptor and donor labeled oligonucleotides are provided linked with

organic linkers to two additional suitable oligonucleotides respectively complementary to the acceptor and donor labeled oligonucleotides labeled at or near their 5' ends with two quenchers respectively so that the quenchers can quench the acceptor and the donor.

18. The method as claimed in any one of claims 1 to 8 wherein at least the oligonucleotide labeled with the acceptor is provided in a hairpin quenched configuration, where the acceptor is provided quenched with a quencher.

19. The method as claimed in any one of claims 1 to 8 wherein both the donor as well as the acceptor labeled oligonucleotides are provided in hair-pin quenched configuration so that both the donor and the acceptor moieties are provided quenched with two separate quenchers, the quenchers are provided on the same oligonucleotides as the donor and acceptor MET moieties and attached at the respective 5' ends.

20. The method as claimed in any one of claims 9-17 wherein the quencher is a non- radiative quencher.

21. The method as claimed in any one of claims 1 to 8 wherein
(a) Atleast the oligonucleotide labeled with the acceptor is provided in quenched configuration by any of the means mentioned in the claims 14 to 18 so that the acceptor remains quenched when the acceptor labeled oligonucleotide is not incorporated into or not hybridized to the amplification product, thus reducing the background and remains unquenched in open configuration of the oligonucleotide producing signal when incorporated into or hybridized to the amplification product,
(b) the amplified sample is illuminated with light absorbed by the donor MET moiety, and
(c) monitoring the sensitized emission from the acceptor and optionally emission from donor of the MET pair moieties.

22. The method as claimed in anyone of claims 1 to 6 and 9 - 13 wherein the size of the amplification product or the nested amplification product as the case may be is of the size of the first oligonucleotide plus the size of the second oligonucleotide plus another zero to twenty-five bases.

23. The method as claimed in anyone of claims 1 to 2 and 4-8 wherein the size of the amplification product or the nested amplification product as the case may be is of the size of the first oligonucleotide plus the size of the second oligonucleotide plus another zero to fifteen bases.

24. The method as claimed in any one of the claims 1 to 6 and 8 - 12 wherein said step of amplifying target sequence comprise a nucleic acid amplification reaction carried out using the above two labeled oligonucleotides as the forward and reverse primers of the amplification reaction, one of the said primers being labeled with a donor MET moiety at or near 3'end and the other primer being labeled with an acceptor MET moiety of a MET pair at or near 3' end such that upon successful amplification of the target sequence both the labeled primers get incorporated into the two strands of the amplification product thus bringing the donor and the acceptor MET moieties within a distance of 2-20 nucleotides of one another resulting in MET, between the two moieties with said amplification reaction; illuminating the reaction mixture with donor excitation radiation or light and measuring the acceptor emission and optionally measuring reduction in donor emission.

25. The method as claimed in claim 24 wherein the donor & acceptor come at a distance of 4 -15 nucleotides.

26. The method as claimed in anyone of claims 1 to 21 wherein a first oligonucleotide of hairpin configuration labeled with a donor moiety at or near its 3' end and with an acceptor moiety capable of absorbing the energy or light emitted by the donor, where the acceptor is selected to be a fluorophore or a non-radiative quencher preferably a quencher including DABCYL or its analogue or nanogold particle, not excluding others at or

near its 5' ends and a second oligonucleotide singly labeled at or near its 3' end also with an acceptor moiety capable of absorbing the energy or light emitted by the donor, where the acceptor is selected from a fluorophore or a quencher preferably a quencher including DABCYL or its analogue or nanogold particle, not excluding others; the first and second oligonucleotides being the two primers of nucleic acid amplification reaction are used such that the emission of the donor is quenched by the quencher/acceptor on the second oligonucleotide only in case of formation of primer dimer but in case of specific amplification product formation the above said quencher/acceptor of second oligonucleotide remains at least 10 bases away from the donor moiety incorporated into the amplification product through the first oligonucleotide and at the same time the quencher of first oligonucleotides remains at least 10 bases away from the donor moiety incorporated into the amplification product through the first oligonucleotides thus allowing the donor moiety to emit its characteristic energy or light and signal generation for the detection or quantitation of a target nucleic acid sequence with increased signal to noise ratio.

27. The method as claimed in any one of the claims 1 to 21 wherein the first primer labeled with donor is provided in quenched configuration by using a complementary oligonucleotide labeled with a quencher for donor separately or linked through an organic linker or otherwise and the second primer is provided labeled with the acceptor in such a way that the donor of the first primer and the acceptor (or quencher) of the second primer are placed at least 10 nucleotides away from each other in specific amplification product thus resulting in signal generation by the donor MET moiety in case of amplification product formation and the same donor and acceptor (or quencher) come close to each other under MET distance in case of primer dimer formation thus quenching the donor emission from primer dimer if any formed during amplification reaction and reducing noise.

28. The method as claimed in any one of the claims of 26 and 27 wherein the quenchers /acceptor remain 10-40 nucleotides away from the donor MET moiety in case of specific amplification product formation

29. The method as claimed in claim 7 wherein said step of amplifying the target sequence comprise a nucleic acid amplification reaction carried out using one . labeled oligonucleotide as one of.the two amplification primers of the target sequence amplification reaction along with the other unlabeled primer and a third labeled oligonucleotide for the amplification product, the said labeled oligonucleotide primer being labeled at or near 3'end with a donor or an acceptor MET moiety of a donor-acceptor MET pair and the said third oligonucleotide being labeled at or near 3' end respectively with an acceptor or donor MET moiety of the above MET pair such that upon successful amplification of the target sequence the labeled primer gets incorporated into one of the two strands of the amplification product and the third labeled oligonucleotide hybridizes to this strand of the amplification product into which the labeled oligonucleotide primer got incorporated thus bringing the donor and the acceptor MET moieties within a MET distance 0-20 nucleotides of one another resulting in MET between the two moieties; the above said amplification reaction comprising the steps of adding . polymerase, reaction buffer, deoxy nucleoside triphosphates in addition to the effective amounts of the amplification primers and the third oligonucleotide to the samples, cycling the sample between at least a denaturation temperature and an elongation temperature, exciting the reaction mixture with the donor exciting radiation or light, measuring the emission of the acceptor MET moiety and optionally the reduction in donor emission, thus allowing detection of nucleic acid target without creating inhibition to amplification reaction and signal measurement without loss of signal.

30. The method as claimed in claim 29 wherein the donor and acceptor MET moieties come at a distance of one to fifteen nucleotides.

31. The method as claimed in any one of claims 1-30 wherein said oligonucleotides are of the length 10 to 50 bases preferably 15 -35 bases.

32. The method as claimed in anyone of claims 1-30 wherein said hair–pin oligonucleotides comprise anyone of the following:

a. a first oligonucleotide 10 – 50 bases long preferably 15 – 35 bases long fully complementary to the target nucleic acid sequence at the 5' end of which is attached a 5 – 9 bases long second oligonucleotide which may or may not be partially or fully complementary to the target sequence but fully complementary to the 3' end of the first oligonucleotide thus forming a stem and loop structure.

b. a first oligonucleotide of length between 15 – 50 preferably 15 – 35 bases fully complementary to the target nucleic acid sequence at the 5' end of which is attached a second oligonucleotide of length 2 to 12 bases which may or may not be complementary to the target sequence and again at the 5' end of the second oligonucleotide is attached a third oligonucleotide of length 5 – 9 bases, the second and the third oligonucleotide may or may not be partly or fully complementary to the target nucleic acid sequence but the third oligonucleotide being fully complementary to 5 – 9 bases at or near the 3' end of the first oligonucleotide thus forming stem and loop structure.

c. a first oligonucleotide of length between 15 – 50 bases preferably between 15 – 35 bases fully complementary to the target nucleic acid sequence at the 5' end of the said first oligonucleotide is attached a second oligonucleotide of length 5 – 9 bases and at the 3' end of the said first oligonucleotide is attached a third oligonucleotide of length 5 – 9 bases, the second and the third oligonucleotides being fully complementary to each other, may or may not be fully or partly complementary to the target nucleic acid sequence thus forming a stem and a loop structure of said hair – pin oligonucleotide

d. a first oligonucleotide of length between 15 – 50 preferably 15 – 35 bases fully complementary to the target nucleic acid sequence at the 5' end of which is attached a second oligonucleotide of length 10 to 30 bases preferably 15-25 through a linker the second oligonucleotide may or may not be partly or fully complementary to the target nucleic acid sequence but the second oligonucleotide being complementary to the bases at or near the 3' end of the first oligonucleotide.

33. The method as claimed in anyone of claims 1 to 32 wherein in case of double labeled hair – pin oligonucleotide the MET moieties (donor/acceptor) and the quencher are attached to the two opposite strand of the hair – pin stem, within the distance of 5 nucleotides but preferably on the two nucleotides opposite to each other.

34. The method as claimed in anyone of claims 1 to 32, wherein, in the event of formation of hair – pin stem structure the nucleotide to which the donor or the acceptor moiety is attached is complementary and opposite to the nucleotide to which the quencher is attached.

35. The method as claimed in anyone of claims 1 to 32, wherein, in the event of hair-pin formation, the nucleotide to which the donor or the acceptor moiety is attached and the complement of the nucleotide to which the quencher is attached are within five nucleotides.

36. The method as claimed in any one of claims 1 to 31 wherein said oligonucleotides are selected from DNA or RNA or chimeric mixtures or derivatives or modified versions thereof adapted for priming the amplification reaction.

37. The method as claimed in anyone of claims 1 to 32, wherein in case of doubly labeled hair-pin oligonucleotide, the nucleotide of the oligonucleotide to which the acceptor moiety or the donor moiety is

attached and nucleotide, to which the quencher is attached, are at least 10 nucleotides apart.

38. The method as claimed in claim 7 for heterozygous mutation detection comprising two amplification primer oligonucleotides one being labeled with a donor moiety near 3' end and two oligonucleotides complementary to each other, one labeled with a first acceptor moiety at or near 3' end and the other labeled with a second acceptor moiety at or near 5' end, the two acceptor moieties and their emission wavelengths being such that two moieties can be detected without much interference.

39. The method as claimed in claim 38 wherein the said acceptor labeled oligonucleotides are provided in quenched configuration.

40. The method as claimed in anyone of claims 1 to 37 other than the claims 7,29 and 30, wherein said labeled oligonucleotides are amplification primers (forward and reverse) of polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), allele specific PCR, methylation status PCR, in – situ PCR, Triamplification, Nucleic acid sequence based amplification, immuno PCR and not excluding others.

41. The method as claimed in claims 1 – 6 and 8 – 28 for he terozygous mutation detection comprising two amplification primer oligonucleotides of the invention one being labeled with a donor MET moiety near 3' end and the other being labeled with an acceptor MET moiety near 3' end, carrying out target amplification reaction and thermal denaturation analysis of the amplification product or products thus amplified and the same method where the labeled oligonucleotides are also provided in quenched configuration.

42. The method as claimed in anyone of claims 1 to 40 wherein one of the said labeled oligonucleotides, the first labeled oligonucleotide is one of the said two amplification primers of many polymerase chain reactions (PCRs) and the second labeled oligonucleotide hybridize to the strand of the

amplification product into which the first labeled oligonucleotide is incorporated.

43. The method as claimed in anyone of claims 1 to 40 used in RNA expression profiling by simultaneously quantitating large number of m-RNAs or C-DNAs using PCR, RT – PCR and not excluding others.

44. The method as claimed in anyone of claims 1 to 43 used in high throughput nucleic acid amplification reactions including PCR, RT-PCR and not excluding others.

45. The method as claimed in anyone of claims 1 to 44used in RNA splice variant detection, wherein the target nucleotide sequence is a m-RNA or a C-DNA and the labeled oligonucleotides of the invention are either amplification primers (forward and reverse) of many nucleic acid amplification reactions including polymerase chain reaction (PCR), Reverse transcription polymerase chain reaction (RT-PCR) etc and not excluding others, one from 3' end of one exon and the other from 5' end of the adjacent exon. Or one of the two amplification primers of many nucleic acid amplification reactions including PCR, RT-PCR and not excluding others from 3' end of one exon and a probe complemantary to 5'end of the adjacent exon.

46. The method as claimed in anyone of claims 1 to 45 wherein the detectable signal emitted by the acceptor MET moiety is sizeable and more intense than the signal emitted by the same if there is no MET.

47. The method as claimed in anyone of claims 1 to 46 wherein the oligonucleotides are so designed that MET moieties come in right proximity such that MET between donor and acceptor moieties can occur.

48. The method as claimed in anyone of claims 1 to 47 wherein FRET is a preferred form of MET.

49. The method as claimed in anyone of claims 1 to 48 wherein said oligonucleotides are deoxy oligonucleotides or peptide nucleic acid or modified oligonucleotides.

50. The method as claimed in anyone of claims 1 to 49 wherein the target nucleic acid sequence is genomic DNA, m-RNA, RNA, c- DNA, chemically synthesized DNA or RNA,

51. The method as claimed in anyone of claims 1 to 50 wherein the target nucleic acid sequence is the sequence of infectious disease agent.

52. The method as claimed in anyone of claims 1 to 51 wherein the target nucleic acid sequence is a human genomic sequence, mutation of which is implicated to the presence of a human disorder or disease.

53. The method as claimed in anyone of claims 1 to 52 wherein the target nucleic acid sequences is a human genomic sequence, the presence or absence of which is implicated to a human disorder or disease.

54. The method as claimed in anyone of claims 1 to 53 wherein the target nucleic acid sequence is a human genomic sequence, the presence or absence of which is implicated to susceptibility to an infectious agent.

55. The method as claimed in anyone of claims 1 to 54 wherein the target nucleic acid sequence is a plant genomic sequence the presence or absence of which is implicated to genotyping of the plants.

56. The method as claimed in anyone of claims 1 to 55 wherein the target nucleic acid sequence is genomic sequence of an infectious agent, the presence or absence of which is implicated to strain typing.

57. The method as claimed in anyone of claims 1 to 56 wherein the target nucleic acid sequence is an amplification product.

58. The method as claimed in anyone of claims 1-36 wherein FRET is the preferred form of MET

59. The method as claimed in anyone of claims 1-36 wherein the donor and acceptor pair moieties are selected from any of the donor - acceptor MET \ FRET pairs known in the art but not limited to those.

60. The method as claimed in anyone of claims of 58 and 59 the donor moiety is selected from the group consisting of but not limited to fluorescein, carboxyfluoroscein (FAM), coumarin, 5-(2' amino ethyl) amino napthalen – 1-sulphonic acid(EDANS), rhodamine, anthranilamide, Reactive Red-4,europium and terbium chelate derivatives and the said acceptor moiety is selected from the group consisting of but not limited to fluorescein, fluorescein derivatives like JOE and others, ethidium, texas red, eosin nitrotyrosine, malachite green, pyrene butyrate, Cy- 3 dye, Cy- 5 dye , DABCYL, DABCYL derivatives, rhodamine, rhodamine derivatives, nanogold.

61. The method as claimed in anyone of claims 10 to 60 wherein the quencher is selected from the group consisting of DABCYL and its derivatives, rhodamine, nanogold particles and many other acceptor moieties.

62. The method as claimed in one of the claims 24 and 25 wherein the acceptor or quencher on the second oligonucleotide absorbs the light of donor on first oligonucleotide and quenches donor emission only in case of formation of primer dimer.

63. The method as claimed in anyone of claims 1 to 60 wherein the donor moiety is a combination of an organic moiety having a large extinction coefficient of absorption and a fluorophore preferably of high quantum yield.

64. The method as claimed in anyone of claims 1 to 40 wherein 6 to 9 bases at or near 3' end form hair-pin structure with the 6 to 9 bases at the 5' end of the oligonucleotide or oligonucleotides, the 6 to 9 bases at the 5' end of the oligonucleotides or oligonucleotide may or may not be complementary to the

target sequence but fully complementary to the 6 to 9 bases at or near 3' end of the oligonucleotide or oligonucleotides.

65. The method as claimed in anyone of claims 1 to 40 wherein 7 to 9 bases at or near 3' end form hair-pin structure with the 7 to 9 bases at the 5' end of the oligonucleotide or oligonucleotides, the 7 to 9 bases at the 5' end of the oligonucleotides or oligonucleotide may or may not be complementary to the target sequence but fully complementary to the 7 to 9 bases at or near 3' end of the oligonucleotide or oligonucleotides.

66. The method as claimed in anyone of claims 1 to 35 used in high throughput nucleic acid amplification, detection and quantitation.

67. The method as claimed in anyone of claims 5 to 9 and 20 to 21 wherein the detection and/ or quantitation of amplified target nucleic acid is accomplished by providing double stranded DNA binding fluorescent dye selected from the group consisting of but not limited to ethidium bromide, CYBER TM GREEN I, pico green, acridine orange, thiazole orange Yo PRO-1 and chromomycin A3.

68. The method as claimed in anyone of claims 5 to 8 wherein the detection and /or quantitation of amplified target nucleic acid sequences is accomplished by providing one of the two oligonucleotides as MET/FRET primer, i.e. labeled with a donor moiety and an acceptor moiety at or near two ends (5' & 3'), acceptor moiety being a fluorophore or quencher.

69. The method as claimed in anyone of claims 5 – 8 and 20 to 21 wherein the detection and /or quantitation of amplified target nucleic acid sequences is accomplished by providing the first oligonucleotide primer being labeled with a binding moiety like biotin, or magnetic particle or microsphere or a hapten or the like or attached to an anchor oligonucleotide directly or through a linker but not excluding others, which can be respectively captured by streptavidin or magnet or centrifugation or anti-hapten antibody, capture oligonucleotide or the like and the second oligonucleotide primer being labeled with a signaling moiety like a fluorophore, rare earth metal chelate, biotin or a

hapten, the hapten being detected utilizing antihapten antibody-enzyme conjugate, steptavidin- enzyme conjugate and enzyme substrate, and other conjugates or by using unlabeled second oligonucleotide primer and providing fluorescently labeled nucleotide in the reaction mixture.

70. The method as claimed in anyone of claims 1 to 69 wherein the higher signal to noise ratio is achieved by applying hair-pin quenched labeled oligonucleotides of the invention in ligase chain reaction.

71. The method as claimed in anyone of claims 1 to 70 wherein the higher signal to noise ratio improvement is achieved by applying hair-pin quenched labeled oligonucleotides of the invention in the detection of a nucleic acid target sequence using MET/FRET between donor & acceptor moieties on two oligonucleotide designed against one strand of the target sequence.

72. The method as claimed in anyone of claims 1 to 71 comprising heterogenous phase detection wherein one of the oligonucleotides is fixed covalently through 5' end or through an internal nucleotide to a solid support through a linker or linker and spacer.

73. The method as claimed in anyone of claims 1 to 72 wherein said solid support to which the labeled oligonucleotide is attached is non – porous and transparent or translucent and preferably glass or plastics like polystyrene, polyethylene, polypropylene or dextran and the like.

74. The method as claimed in anyone of claims 1 to 73 wherein one or both the amplification primers are labeled at or near 3' end with acceptor moiety or moieties and one of the four deoxynucleotides is provided labeled with the donor moiety in appropriate concentrations and composition, and wherein on incorporation of the acceptor labeled primer or primers and the donor labeled nucleotide into the amplification product there is MET/FRET between the donor and the acceptor.

75. The method as claimed in anyone of claims 1 to 74 wherein one or both the primers are provided labeled with a donor or acceptor moiety and a double stranded DNA intercalating dye suitable to act as an acceptor or donor respectively whereby on successful amplification donor/acceptor labeled primer/primers get incorporated into the amplification product and the double stranded DNA binding (intercalating) dye get intercalated into the amplification product thus bringing it close to the donor or acceptor moiety as the case may be and resulting in MET/FRET which can be measured.

76. The method as claimed in anyone of claims 1 to 75 wherein fluoresecin labeled primer and double stranded DNA binding dye Ethidium bromide are used, where fluorescein act as donor and ethidium act as acceptor for FRET to take place between the two.

77. The method as claimed in any of the claims 1-76 wherein relates to real time target sequence detection or quantitation.

78. The method as claimed in any one of claims 1 to 76 wherein the oligonucleotides used are selected from:

1). 5'-GGG GTA CTA CAG CGC CCT GA – 3'

2). 5'-GGG GTA CTA CAG CGC CCT GA –3'

FAM

3). 5'-GTC CTG GAA GAT GGC CAT GG –3'

4). 5'-GTC CTG GAA GAT GGC CAT GG –3'

Joe

5). 5'-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

6). 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG -3'

Joe

7). 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

FAM

8). 5'-GCT CAT GGC GCC TGC CTG G –3'

DABCYL

9). 5'-DABCYL-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

Joe

10).5'-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

11).5'-DABCYL-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

FAM

79. A kit for use in method of analogous detection and / or quantitation of target nucleic acid sequence or sequences present in a sample comprising

(a) a polymerase or polymerases

(b) a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence suitably labeled with a donor MET/FRET moiety at or near 3' end.

(c) a second oligonucleotide of sequence at 5' end of the first nucleotide sequence complementary to the nucleotide sequence flanking the target nucleotide sequence or the segment of the target nucleotide sequence suitably labeled with an acceptor MET/FRET moiety at or near 3' end.

(d) deoxy nucleotides in solution (water or buffer) or lyophilized.

(e) a reaction buffer for the nucleic acid amplification reaction.

wherein the first and the second oligonucleotide sequences comprise the two primers (forward and reverse) of many nucleic acid

amplification reactions, and adapted to generate a detectable signal if the two oligonucleotides get incorporated into two opposite strands of amplified product and come in right proximity.

80. The kit as claimed in claim 77 wherein the first oligonucleotide contains a donor MET/FRET moiety attached to a nucleotide at or near 3' end that is capable of emitting energy or light.

81. The kit as claimed in anyone of claims 77 and 78 wherein the second oligonucleotide contains an acceptor MET/FRET pair moiety attached to a nucleotide at or near 3' end that is capable of absorbing an amount of the energy or light emitted by the donor and capable of emitting energy or light.

82. The kit as claimed in anyone of claims 77 to 79 adapted to emit a detectable signal is emitted from the acceptor moiety on illumination of the reaction mixture by the donor specific exciting light.

83. The kit as claimed in anyone of claims 77 to 80 adapted to emit a detectable signal emitted by the acceptor moiety labeled oligonucleotide more intense than signal emitted by the same oligonucleotide when MET/FRET does not take place.

84. The kit as claimed in claim 77 or 81 adapted to generate a substantial signal.

85. The kit as claimed in anyone of claims 77 to 82 wherein the acceptor labeled oligonucleotide is adapted to emit detectable signal only when MET/FRET takes place and the two oligonucleotides come in appropriate proximity for MET/FRET to take place.

86. The kit as claimed in anyone of claims 77 to 83 wherein the oligonucleotides are oligodeoxynucleotides and are either linear or of hairpin configuration.

87. The kit as claimed in anyone of claims 77 to 84 wherein the oligonucleotides are oligodeoxynucleotides and are of hairpin configuration, linear or other configurations as claimed in claims 1 – 36.

88. The kit as claimed in anyone of claims 77 to 85 wherein the polymerase is a DNA polymerase.

89. The kit as claimed in anyone of claims 77 to 86 wherein the polymerase is a reverse transcriptase.

90. The kit as claimed in anyone of claims 77 to 87 wherein the polymerases are a reverse transcriptase and a DNA polymerase.

91. The kit as claimed in anyone of claims 77 to 88 comprising multiple oligonucleotide pairs as claimed in anyone of claims 1 to 12 for detection and /or quantitation of multiple target sequences.

92. The kit as claimed in anyone of claims 77 to 89 further comprising a third oligonucleotide complementary to the second oligonucleotides containing at or near its 5' end a quencher for the acceptor moiety on the second oligonucleotide, so that the second and the third oligonucleotides remain hybridized to each other when the second oligonucleotide is not incorporated into the amplification product thus keeping background emission low, signal is generated from separation of second and third oligonucleotides and energy transfer between the donor moiety on the first oligonucleotide and the acceptor moiety on the second oligonucleotide.

93. The kit of claim 90 wherein additionally a fourth oligonucleotide complementary to the first oligonucleotide and containing at or near its 5' end a quencher for the donor so that the first and fourth oligonucleotides hybridized to each other where the first oligonucleotide is not incorporated into the amplification product thus keeping background emission further low, signal is generated from separation of second and third oligonucleotides, first oligonucleotide and fourth oligonucleotide, and energy transfer between the

donor moiety on the first oligonucleotide and the acceptor moiety on the second oligonucleotide on incorporation of the first and second oligonucleotides into the amplification product.

94. A kit as claimed in any one of claims 77 – 91, wherein oligonucleotides or multiple pairs of oligonucleotide are provided separately.

95. A kit for use in method of analogous detection and/ or quantitation of target nucleic acid sequence or sequences present in a sample comprising:

a. A polymerase or polymerases.
b. A first oligonucleotide of nucleotide sequence complementary to the nucleotide sequence flanking a target nucleotide sequence suitably labeled with a donor MET/FRET moiety near 3' end.
c. A second oligonucleotide sequence at 5' end of the first nucleotide sequence suitably labeled with an acceptor MET/FRET moiety near 3' end.
d. Deoxy nucleotides in solution (water or buffer) or lyophilized.
e. Reaction buffer for amplification reaction.

96. A kit as claimed in claim 93 for the detection of nucleic acid target or targets using the methods as claimed in any of the claims 1 to 4 and 8 to 17.

97. The kit as claimed in claim 94 wherein the second oligonucleotide contains a third oligonucleotide sequence (5-9 bases long) at the 5' end of the second oligonucleotide, which is fully complementary to the second oligonucleotide sequence and the third oligonucleotide contains at or near its 5' end a quencher for acceptor in order to keep the acceptor quenched for reducing background, when the second oligonucleotide is not incorporated into the amplification product.

98. The kit as claimed in claim 93 or 95 wherein the first and the second oligonucleotide sequences are the two primers (forward and reverse) of nucleic acid amplification reaction and a detectable signal is generated if the

two oligonucleotides get incorporated into the two opposite strands of the amplification product and come in right proximity.

99. The kit as claimed in anyone of claims 93 to 96 wherein the first oligonucleotide contains a donor MET/FRET moiety attached to a nucleotide at or near 3' end that is capable of emitting energy or light.

100. The kit as claimed in anyone of claims 93 to 97 wherein the second oligonucleotide contains an acceptor MET/FRET pair moiety attached to a nucleotide at or near 3' end that is capable of absorbing an amount of the energy or light emitted by the donor and capable of emitting energy or light.

101. The kit as claimed in anyone of claims 93 to 98 wherein the acceptor moiety is adapted to emit a detectable signal emitted from the acceptor moiety on illumination of the reaction mixture by the donor specific exciting light.

102. The kit as claimed in anyone of claims 93 to 99 wherein the acceptor moiety is adapted to emit a detectable signal more intense than a signal emitted by the same oligonucleotide if MET/FRET does not take place.

103. The kit as claimed in anyone of claims 93 to 100 wherein the detectable signal generated is substantial.

104. The kit as claimed in anyone of claims 93 to 101 wherein the acceptor labeled oligonucleotide is adapted to emit detectable signal only when MET/FRET takes place, i.e. the two oligonucleotides come in right proximity for MET/FRET to take place.

105. The kit as claimed in anyone of claims 93 to 102 wherein the oligonucleotides are oligodeoxynucleotides are linear or of hair-pin configuration, or of other configurations as described in claims 1 – 36.

106. The kit as claimed in anyone of claims 93 to 103 wherein the polymerase is a DNA polymerase.

107. The kit as claimed in anyone of claims 93 to 104 wherein the polymerase is a reverse transcriptase.

108. The kit as claimed in anyone of claims 93 to105 wherein the polymerases are a reverse transcriptase and a DNA polymerase.

109. The kit as claimed in anyone of claims 93 to 106 comprising multiple oligonucleotide pairs in any combination of many possibilities of for detection and /or quantitation of multiple target sequences.

110. The kit as claimed in anyone of claims 93 to 107 wherein the first oligonucleotide contains a donor moiety at or near its 3' end and a fourth oligonucleotide of the length 5 – 9 bases, at 5'end of the first oligonucleotide, fully complementary to the first oligonucleotide so that it can form a 5 to 9 base stem and loop structure, the fourth oligonucleotide in turn containing an acceptor moiety preferably a quencher which can absorb the energy or light of the donor, at or near its 5' end so that the donor remains quenched when the first oligonucleotide is not incorporated into the amplification product; and the second oligonucleotide is a linear oligonucleotide suitably labeled with an acceptor moiety preferably a quencher for the donor moiety at or near its 3' end so that the acceptor on second oligonucleotide and the donor on the first oligonucleotide comes within MET/FRET distance in case of primer dimer formation during amplification reaction and remains at least 10 nucleotides away from each other in case of formation of specific amplification product designed for when both the first oligonucleotide and the second oligonucleotide are used as forward and reverse primers for the amplification reaction and both get incorporated into the specific amplification product, i.e. incorporation of both the oligonucleotide primers resulting in an amplification product where both the acceptors remain at least 10 nucleotides away from the donor moiety allowing donor moiety to generate signal on illumination with light for donor.

111. The kit as claimed in anyone of claims 95 and 108 wherein the third oligonucleotide is attached to the second oligonucleotide and the fourth oligonucleotide is attached to first oligonucleotide respectively through a fifth and a sixth oligonucleotide 2 – 12 nucleotides long.

112. The kit as claimed in anyone of claims 93 to 109 wherein the third oligonucleotide contains at its 5' end a suitable organic linker, which is attached to a seventh oligonucleotide complementary to the second oligonucleotide and contains at or near the 5' end of the seventh oligonucleotide a quencher for the acceptor moiety.

113. The kit as claimed in anyone of claims 93 to 110 wherein the first oligonucleotide also contains at its 5'end a suitable organic linker which is attached to an eighth oligonucleotide complementary to the first oligonucleotide and contains a quencher for the donor moiety at or near 5' end of the eighth oligonucleotide.

114. The kit as claimed in anyone of claims 93 to 111 wherein the first oligonucleotide contains at its 5' end a fourth oligonucleotide sequences (5-9 bases long), which is complementary to the first oligonucleotide and contains at its 5' end a quencher for the donor placed near the 3'end of the first oligonucleotide in addition to the second oligonucleotide suitably labeled at or near 3'end with an acceptor moiety and contains at 5'end the third oligonucleotide 5 – 9 bases long, complementary to second oligonucleotide containing a quencher for the acceptor moiety at 5' end of the third oligonucleotide so that both the donor and the acceptor MET/FRET moieties remain quenched when both the first and the second oligonucleotides are not incorporated into the amplification product and a signal is generated when both the first and the second oligonucleotides being the forward and reverse primers of nucleic acid amplification reaction get incorporated into the specific amplification product thus bringing the donor and the acceptor moieties in right proximity for MET/FRET to take place between the donor and the acceptor moieties.

115. The kit as claimed in anyone of claims 93 to 112 wherein additionally positive control template and suitable MET/FRET labeled primers are also included as control for amplification reaction.

116. A Kit for use in method of analogous detection and/or quantitaiton of target nucleic acid sequence or sequences present in a sample comprising

a. polymerase or polymerases.

b. a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence or a segment of a target nucleotide sequence and a second oligonucleotide of sequence at the 5' end of the first oligonucleotide sequence complementary to the nucleotide sequence flanking the target nucleotide sequence or the segment of target nucleotide sequence.

c. a third oligonucleotide of sequence complementary to the above said target nucleotide sequence or the segment of the target nucleotide sequence.

d. deoxynucleotides in solution (water or buffer) or lyophilized.

e. reaction buffer for nucleic acid amplification reaction wherein the first and second oligonucleotide sequences are the two primers (forward and reverse) of nucleic acid amplification reaction.

f. the first oligonucleotide is suitably labeled with a first moiety of a MET/FRET pair at or near 3' end and the third oligonucleotide is labeled at or near 3'end with the second moiety of the MET/FRET pair, and a detectable signal is generated when the first oligonucleotide gets incorporated into one of the two strands of the amplification product and the third oligonucleotide hybridize to the strand of the amplification product into which MET/FRET labeled primer gets incorporated and come in right proximity designed for.

117. The kit as claimed in claim 113 & 114 wherein the MET/FRET pair consists of a donor moiety, and an acceptor moiety capable of absorbing an amount of the energy or light emitted by the donor and capable of emitting energy or light.

118. The kit as claimed in anyone of claims 113 to 115 wherein the MET/FRET pair comprise of a donor moiety, i.e. capable of emitting energy or light and a quencher capable of absorbing energy or light emitted by the donor.

119. The kit as claimed in anyone of claims 114 to 116 wherein the detectable signal is generated only when the MET/FRET takes place and through quenching of donor emission on illumination of the reaction mixture by the donor specific exciting light.

120. The kit as claimed in anyone of claims 114 to 117 wherein said acceptor is adapted to generate a detectable signal on illumination of reaction mixture by the donor specific exciting light only when MET/FRET takes place.

121. The kit as claimed in anyone of claims 114 to 118 wherein the detectable signal emitted by the acceptor moiety labeled oligonucleotide is more intense than a signal emitted by the same oligonucleotide if MET/FRET does not take place.

122. The kit as claimed in anyone of claims 114 to 119 wherein the detectable signal is substantial.

123. The kit as claimed in anyone of claims 114 to 120 wherein the acceptor labeled oligonucleotide is adapted to emit detectable signal only when MET/FRET takes place, i.e. the first oligonucleotide and the third oligonucleotide come in appropriate proximity for MET/FRET to take place.

124. The kit as claimed in anyone of claims 114 to 121 wherein the oligonucleotides are oligodeoxynucleotides and are of hair-pin configuration, linear or of other configurations as claimed in any of the claims 1 – 37.

125. The kit as claimed in anyone of claims 114 to 122 wherein the polymerase is a DNA polymerase.

126. The kit as claimed in anyone of claims 114 to 123 wherein the polymerase is a reverse transcriptase

127. The kit as claimed in anyone of claims 114 to 124 wherein the polymerases are a reverse transcriptase and a DNA polymerase.

128. The kit as claimed in anyone of claims 114 to 125 wherein comprising multiple oligonucleotide sets in any of many possible combination for detection and/or quantitation of multiple target sequences.

129. The kit as claimed in anyone of claims 114 to 126 further comprising a fourth oligonucleotide complementary to the oligonucleotide (first / third) labeled with the acceptor MET/FRET moiety and carrying a quencher at or near 5' end so that the acceptor remains quenched when the acceptor labeled oligonucleotide does not get incorporated into or hybridized to the amplification product; the signal is generated from separation of the quencher labeled oligonucleotide and acceptor labeled oligonucleotide and energy transfer from donor moiety to acceptor moiety.

130. The kit as claimed in anyone of claims 114 to 127 wherein the first oligonucleotide is labeled with a donor moiety and the third oligonucleotide is labeled at or near 3' end with a first acceptor moiety, which can absorb emission energy or light of the donor moiety and can emit energy or light, and the fourth oligonucleotide is labeled at or near 5' end with a second acceptor moiety that can absorb the energy or light emitted by the donor moiety and capable of emitting energy or light, the emission of first and the second acceptor moieties are so chosen that their emissions can be measured separately.

131. The kit as claimed in anyone of claims 114 to 128 wherein additionally positive control template and suitable MET/FRET labeled primers are also included as control for amplification reaction.

132. The kit as claimed in anyone of claims 114 to 129 wherein the first oligonucleotide is labeled near 3' end with a donor MET/FRET moiety and a double stranded DNA intercalating dye capable of absorbing energy or light emitted by the donor moiety and emitting energy or light is also provided.

133. The kit as claimed in anyone of claims 114 to 130 wherein the first oligonucleotide is labeled near 3' end with an acceptor MET/FRET moiety and a double stranded DNA intercalating dye capable of emitting energy or light on illumination is provided such that the acceptor moiety is capable of absorbing the energy or light emitted by the intercalating dye and emitting energy or light.

134. A kit for use in method of analogous (detection) of target nucleic acid sequence or sequences present in a sample comprising.

a. a polymerase or polymerase.
b. a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence or a segment of target nucleotide sequence and a second oligonucleotide sequence at the 5' end of the first oligonucleotide sequence complementary to the nucleotide sequence flanking the target nucleotide sequence or the segment of the target nucleotide sequence.
c. a third oligonucleotide of sequence complementary to the target nucleotide sequence or the segment of the target nucleotide sequence amplified by amplification reaction.
d. deoxynucleotides in solution (water or buffer) or lyophilized.
e. reaction buffer for nucleic acid amplification reaction

wherein the first and second oligonucleotide sequences are the two primers (forward and reverse) of nucleic acid amplification reaction; the first oligonucleotide sequence is suitably labeled at or near 3' end with a donor MET/FRET moiety; the third oligonucleotides sequence is labeled at or near 3' end with an acceptor MET/FRET moiety and carries at its 5' end a fourth oligonucleotide sequence 5-9 nucleotide long fully

complementary to a part of third oligonucleotide sequence so that the third oligonucleotide forms a hair – pin 5 to 9 nucleotide stem and loop structure with the fourth oligonucleotide and the fourth oligonucleotide additionally carries a quencher at or near its 5' end.

135. The kit as claimed in claim 132 adapted to generate a detectable signal is generated when the first oligonucleotide gets incorporated into one of the two strands of the amplification product and the third oligonucleotide hybridize to the strand of the amplification product into which MET/FRET labeled primer gets incorporated and come in right proximity designed for.

136. The kit as claimed in anyone of claims 132 or 133 wherein the acceptor MET/FRET moiety remains quenched when the third oligonucleotide is not hybridized to the target sequence and a signal is generated when the third oligonucleotide hybridize to the target sequence and MET/FRET takes place between the donor and the acceptor MET/FRET moieties.

137. The kit as claimed in anyone of claims 133 to 134 wherein the MET/FRET pair consists of a donor moiety, and an acceptor moiety capable of absorbing an amount of the energy or light emitted by the donor and capable of emitting energy or light.

138. The kit as claimed in anyone of claims 133 to 135 wherein the acceptor moiety is adapted to generate a detectable signal on illumination of reaction mixture by the donor specific exciting light only when MET/FRET takes place.

139. The kit as claimed in claim 136 wherein the detectable signal emitted by the acceptor moiety labeled oligonucleotide is more intense than a signal emitted by the same oligonucleotide if MET/FRET does not take place.

140. The kit as claimed in anyone of claims 136 & 137 wherein the detectable signal is substantial.

141. The kit as claimed in anyone of claims 136 to 138 wherein the acceptor labeled oligonucleotide emits detectable signal only when MET/FRET takes place.

142. The kit as claimed in anyone of claims 133 to 139 wherein the oligonucleotides are oligodeoxynucleotides and are of linear or of other configuration as claimed in any of claims 1 – 37.

143. The kit as claimed in anyone of claims 133 to 140 wherein the polymerase is a DNA polymerase.

144. The kit as claimed in anyone of claims 133 to 141 wherein the polymerase is a reverse transcriptase.

145. The kit as claimed in anyone of claims 133 to 142 wherein the polymerases are a reverse transcriptase and a DNA polymerase.

146. The kit as claimed in anyone of claims 137 to 143 comprising multiple oligonucleotide sets of the claim for detection and/or/quantitation of multiple target sequences.

147. A kit for the detection of target nucleic acid sequences or sequences using the oligonucleotides of claims 5 to 9 wherein the detection and /or quantitation of amplified target nucleic acid sequences is accomplished by providing the first oligonucleotide primer being labeled at or near 5'end with a binding moiety like biotin, or magnetic particle or microsphere or a hapten or the like or attached to an anchor oligonucleotide directly or through a linker but not excluding others, which can be respectively captured by streptavidin or magnet or centrifugation or anti-hapten antibody, capture oligonucleotide or the like and the second oligonucleotide primer being labeled with a signaling moiety like a fluorophore, rare earth metal chelate, biotin or a hapten, the hapten being detected utilizing antihapten antibody-enzyme conjugate, steptavidin- enzyme conjugate and enzyme substrate, and other conjugates

or by using unlabeled second oligonucleotide primer and providing fluorescently labeled nucleotide in the reaction mixture.

148. A kit for the detection of target nucleic acid sequences using the oligonucleotides of claims 5 to 9 wherein the detection and/ or quantitation of amplified target nucleic acid is accomplished by providing double stranded DNA binding fluorescent dye selected from the group consisting of eithidium bromide, CYBER TM GREEN I, pico green, acridine orange, thiazole orange Yo PRO- 1 and chromomycin A3 but not excluding others.

149. A kit for the detection of target nucleic acid sequence or sequences using oligonucleotides of claims 5 to 98 wherein the detection and /or quantitation of amplified target nucleic acid sequences is accomplished by providing one of the two oligonucleotides as MET/FRET primer, i.e. labeled with a donor moiety and an acceptor moiety at or near two ends (5' & 3'), acceptor moiety being a fluorophore or quencher.

150. The kit as claimed in any one of claims 78 to 147 wherein the oligonucleotides used are selected from:

1). 5'-GGG GTA CTA CAG CGC CCT GA – 3'

2). 5'-GGG GTA CTA CAG CGC CCT GA –3'

FAM

3). 5'-GTC CTG GAA GAT GGC CAT GG –3'

4). 5'-GTC CTG GAA GAT GGC CAT GG –3'

Joe

5). 5'-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

6). 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG -3'

Joe

7). 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

FAM

8). 5'-GCT CAT GGC GCC TGC CTG G –3'

DABCYL

9). 5'-DABCYL-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

Joe

10).5'-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

11).5'-DABCYL-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

FAM

151. A method of manufacture of a kit for use in method of analogous detection and / or quantitation of target nucleic acid sequence or sequences present in a sample comprising

(a) Providing a polymerase or polymerases

(b) Providing a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence suitably labeled with a donor MET/FRET moiety at or near 3' end.

(c) Providing a second oligonucleotide of sequence at 5' end of the first nucleotide sequence complementary to the nucleotide sequence flanking the target nucleotide sequence or the segment of the target nucleotide sequence suitably labeled with an acceptor MET/FRET moiety at or near 3' end.

(d) Providing deoxy nucleotides in solution (water or buffer) or lyophilized.

(e) Providing a reaction buffer for the nucleic acid amplification reaction.

wherein the first and the second oligonucleotide sequences comprise the two primers (forward and reverse) of many nucleic acid amplification reactions, and adapted to generate a detectable signal if the two oligonucleotides get incorporated into two opposite strands of amplified product and come in right proximity.

152. A method of manufacture of a kit for use in method of analogous detection and / or quantitation of target nucleic acid sequence or sequences present in a sample as claimed in anyone of claims 78 to 148.

153. A method for analyzing for detection and /or quantitation of a target nucleic acid sequence or sequences of a sample, by nucleic acid amplification reaction and kit or kits for use in such method substantially as herein described and illustrated with reference ``to the accompanying examples and figures and modifications thereof.

Dated this 24th day of May 2002.

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Of S. MAJUMDAR & CO.
Applicants' Agent

ABSTRACT

Disclosure of a method for the detection and quantitation of polynucleotide sequences in a sample of biological or non-biological material through target poly nucleotide sequence amplification by polymerase chain reaction using chemically labeled oligonucleotide amplification primers and formation of an entity between the amplified polynucleotide sequence and chemically labeled polynucleotide having a sequence complementary to the target polynucleotide sequence for determining the identity and /or presence and / or quantitation of the target poly nucleotide sequences. The chemical label covalently attached to the oligonucleotide amplification primer and polynucleotide or oligonucleotide comprise molecular energy transfer labels (donor and acceptor). It is again a very sensitive, rapid and reliable method with better sensitivity, specificity and reliability for the detection of polynucleotide sequence. It also greatly reduces the possibility of amplification product carry-over contamination and adaptable for many formats of nucleic acids amplifications and real time measurements.

To
The Controller of Patents
The Patent Office
At Mumbai.

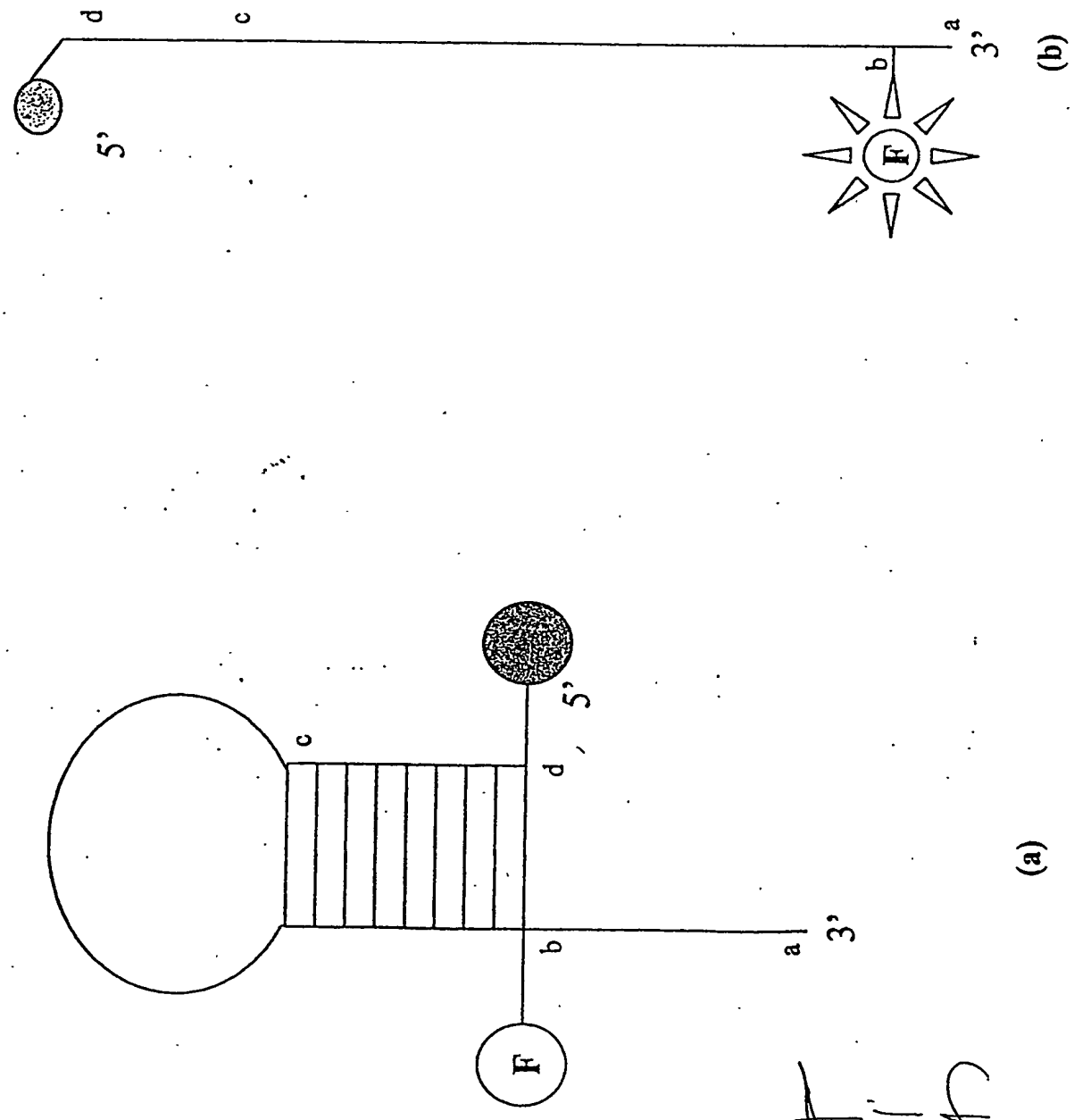

Fig. 1A

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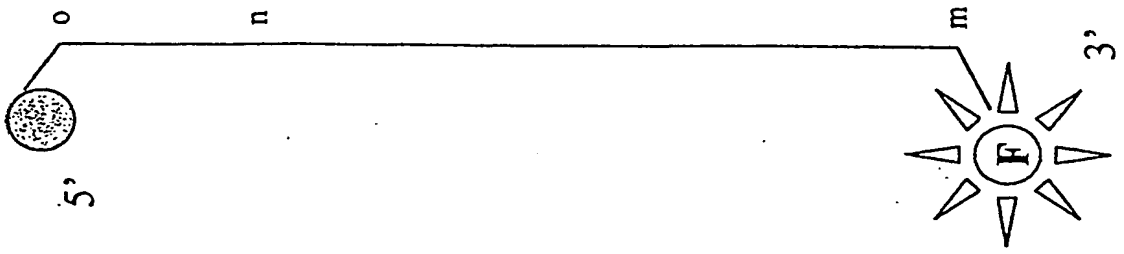
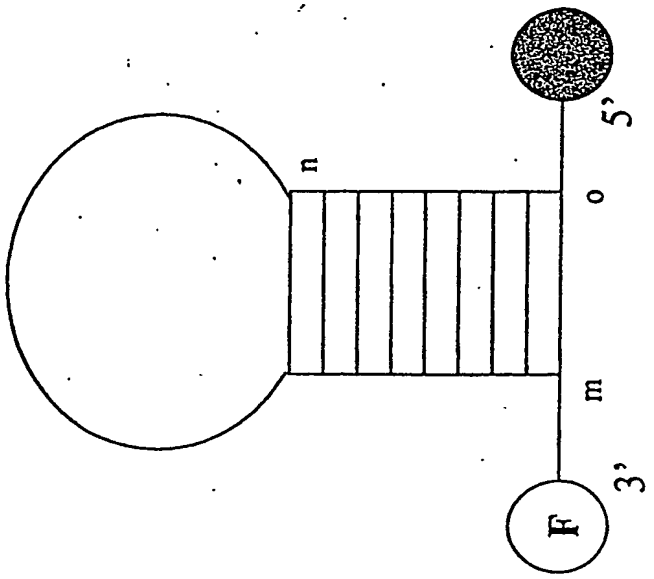


(b)



(a)

Fig. 1B

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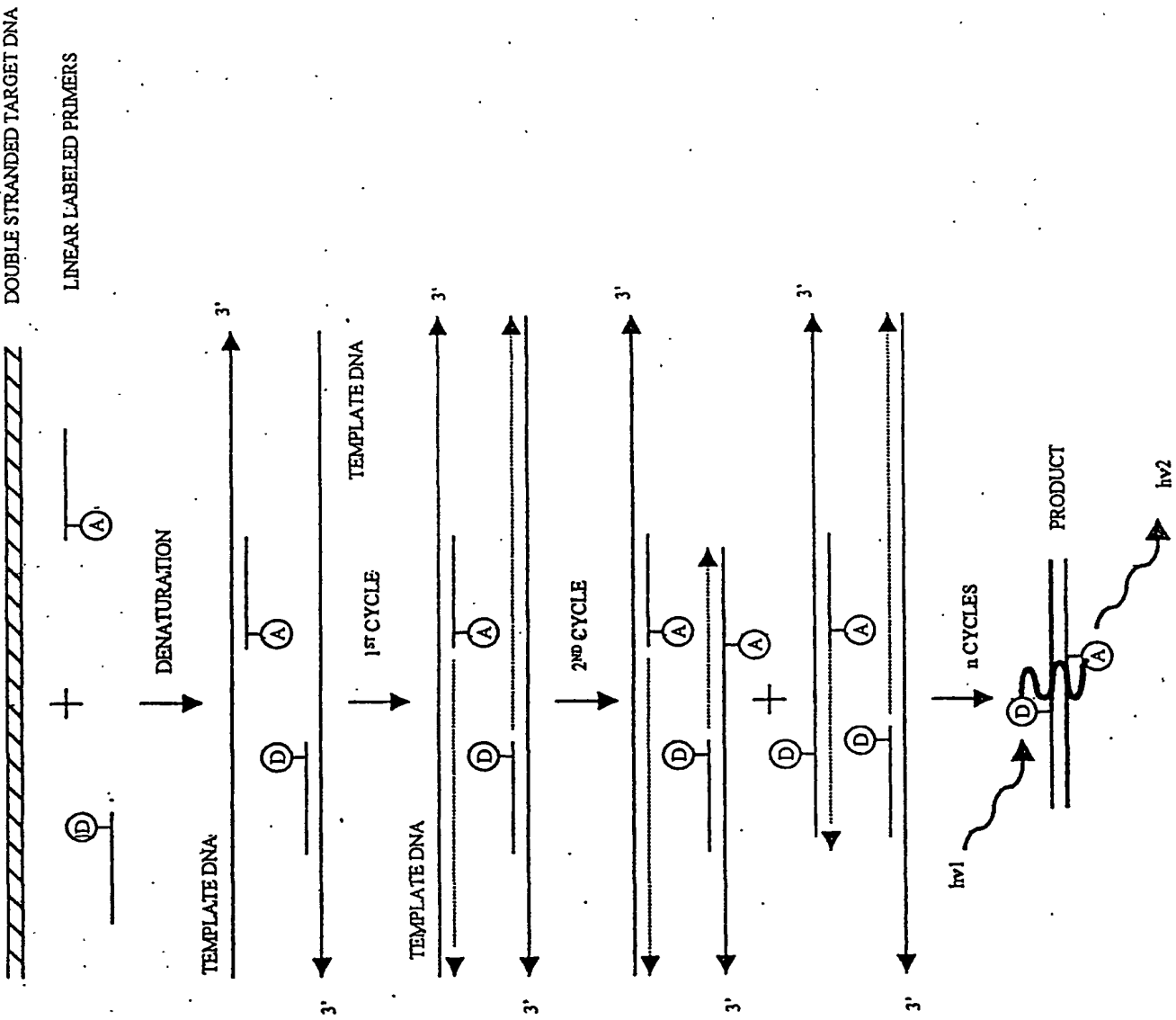

FIG. 2

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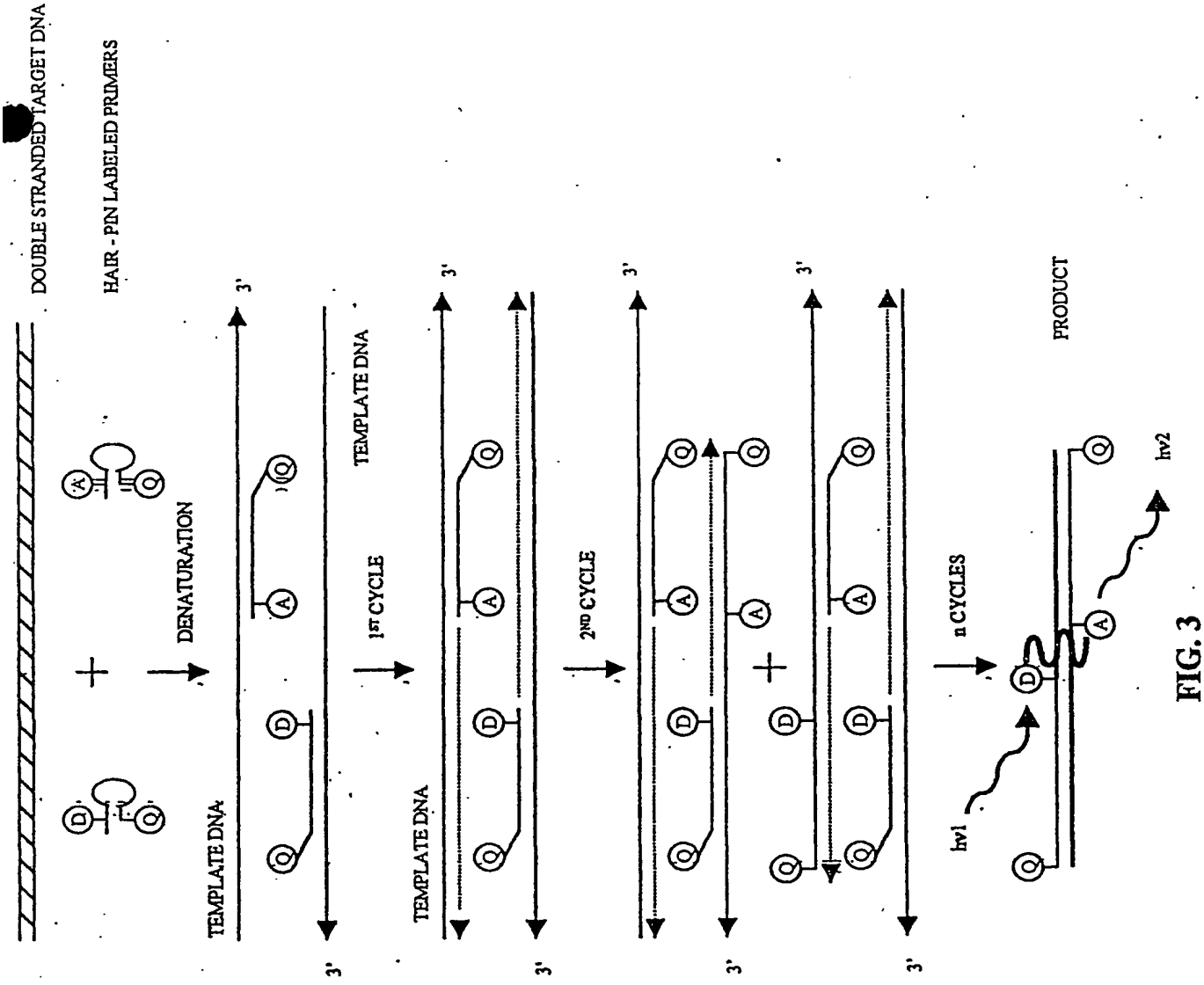

FIG. 3

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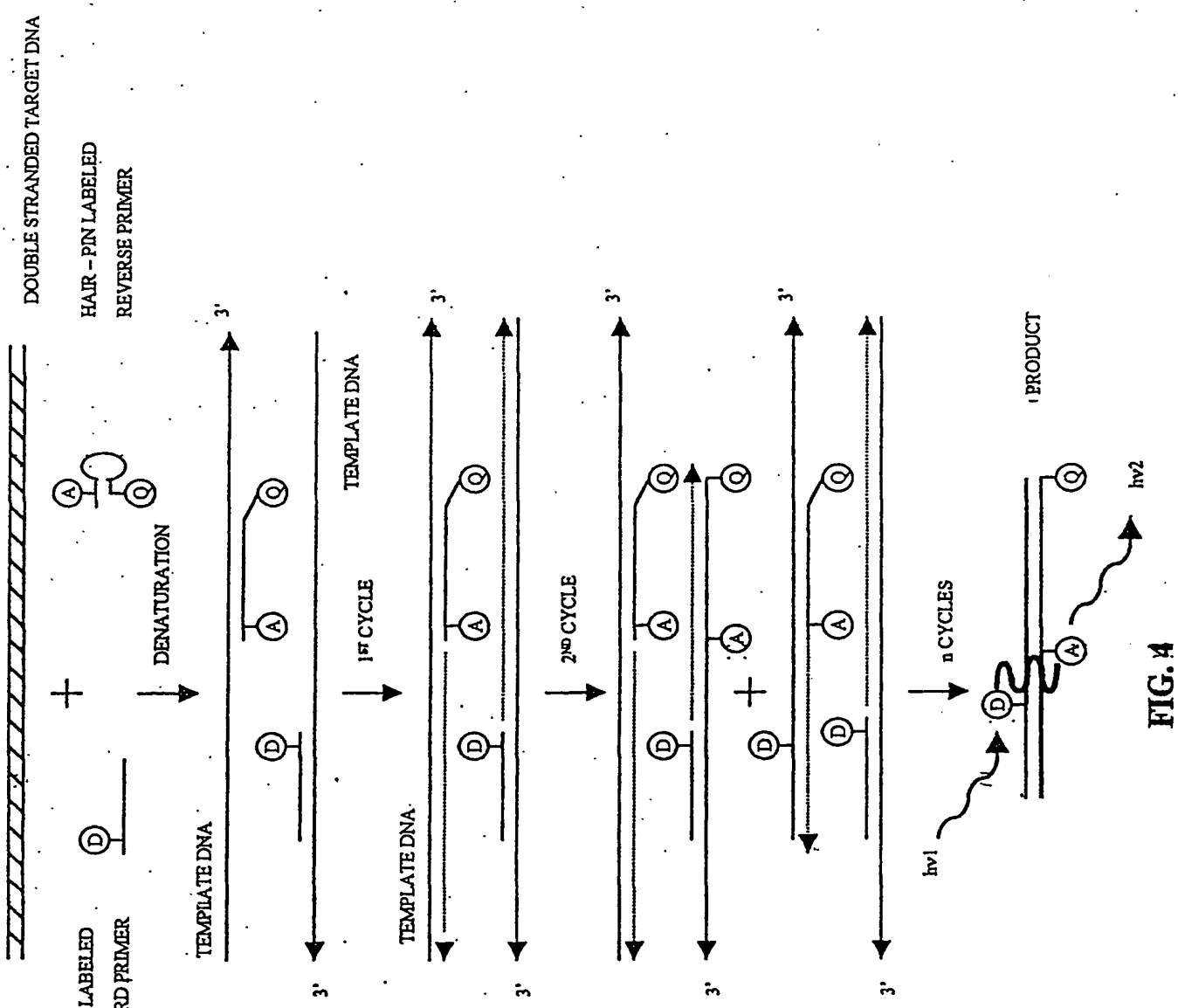

FIG. 4

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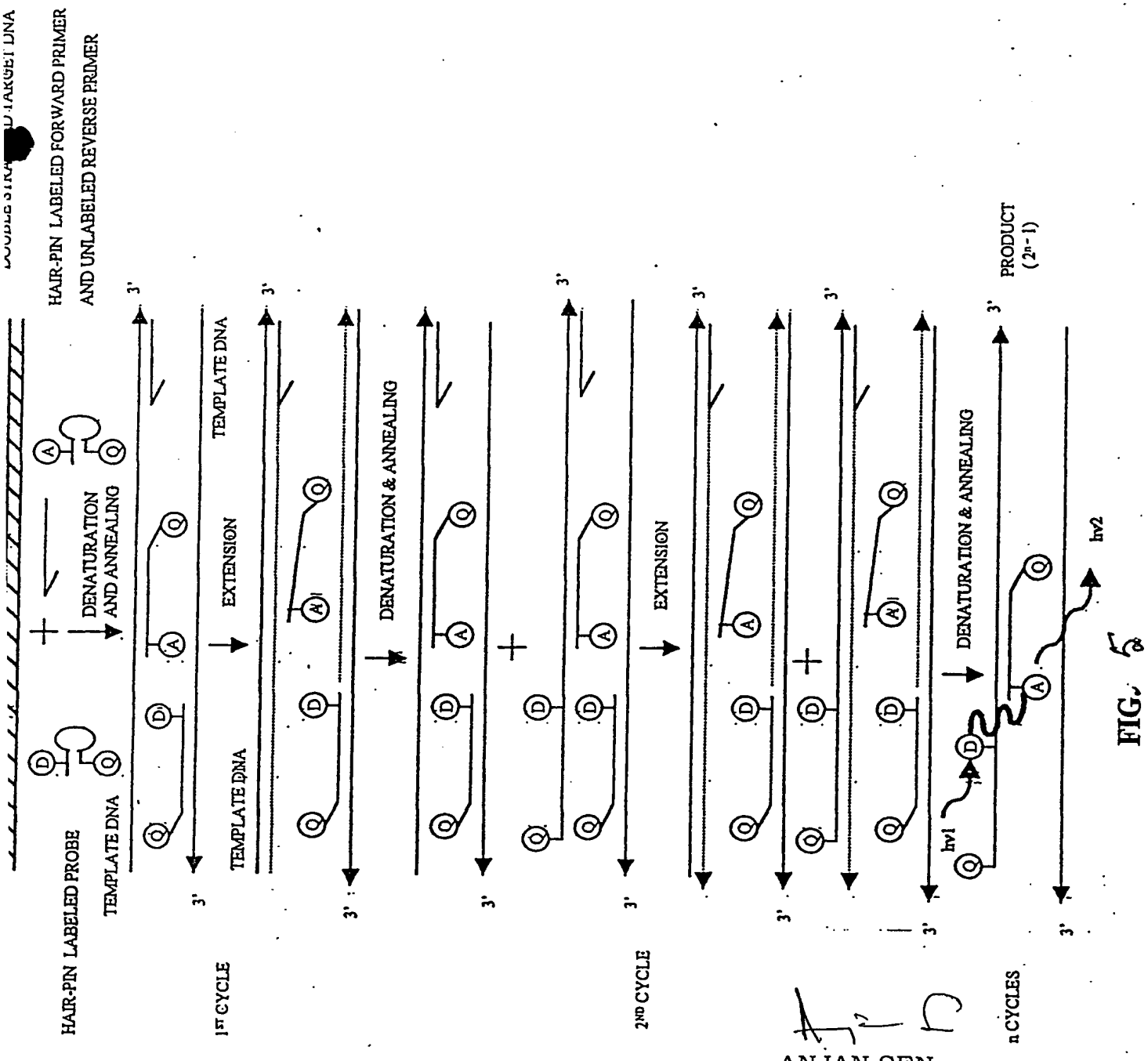

FIG. 5

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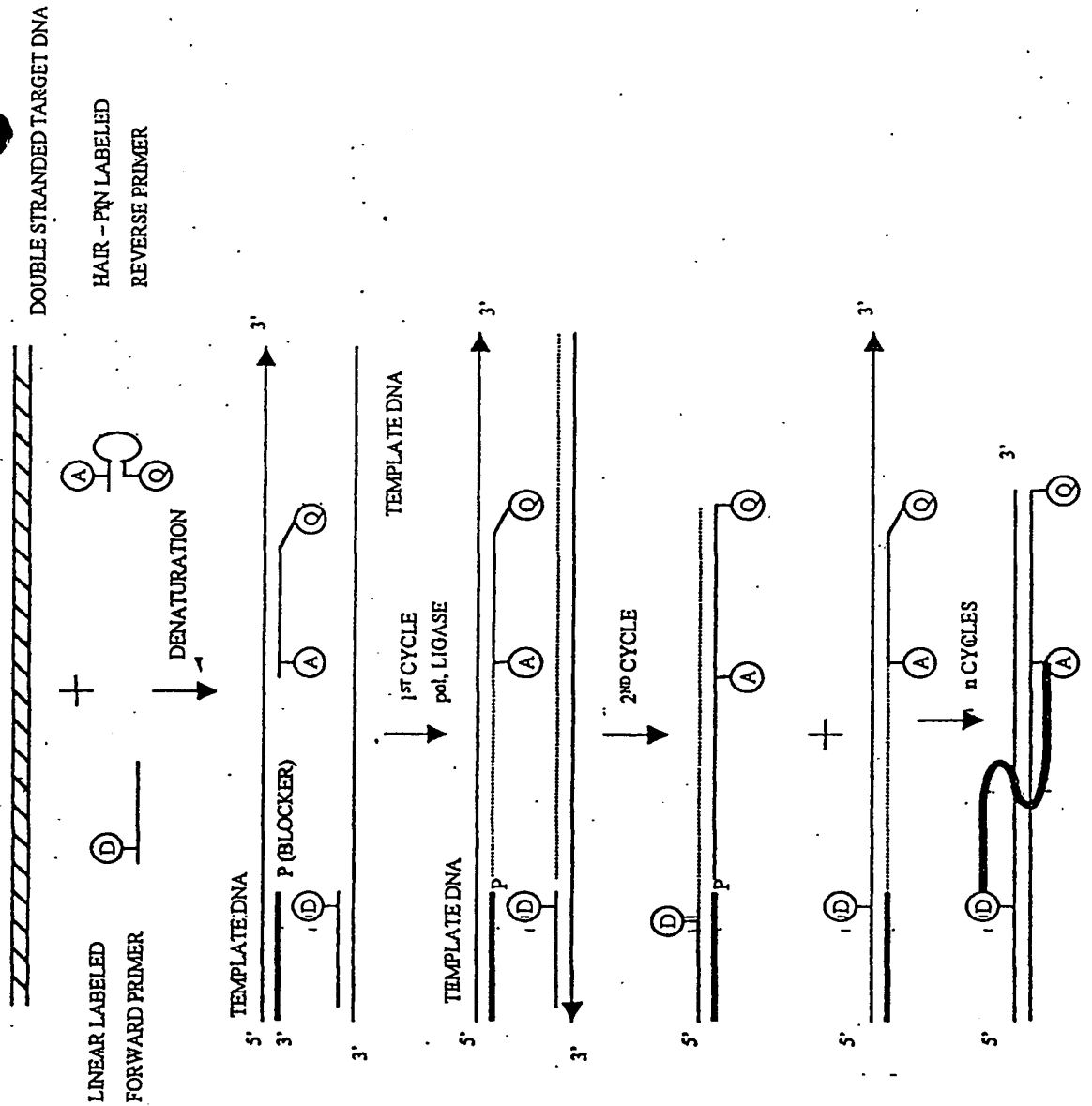

Fig. 6

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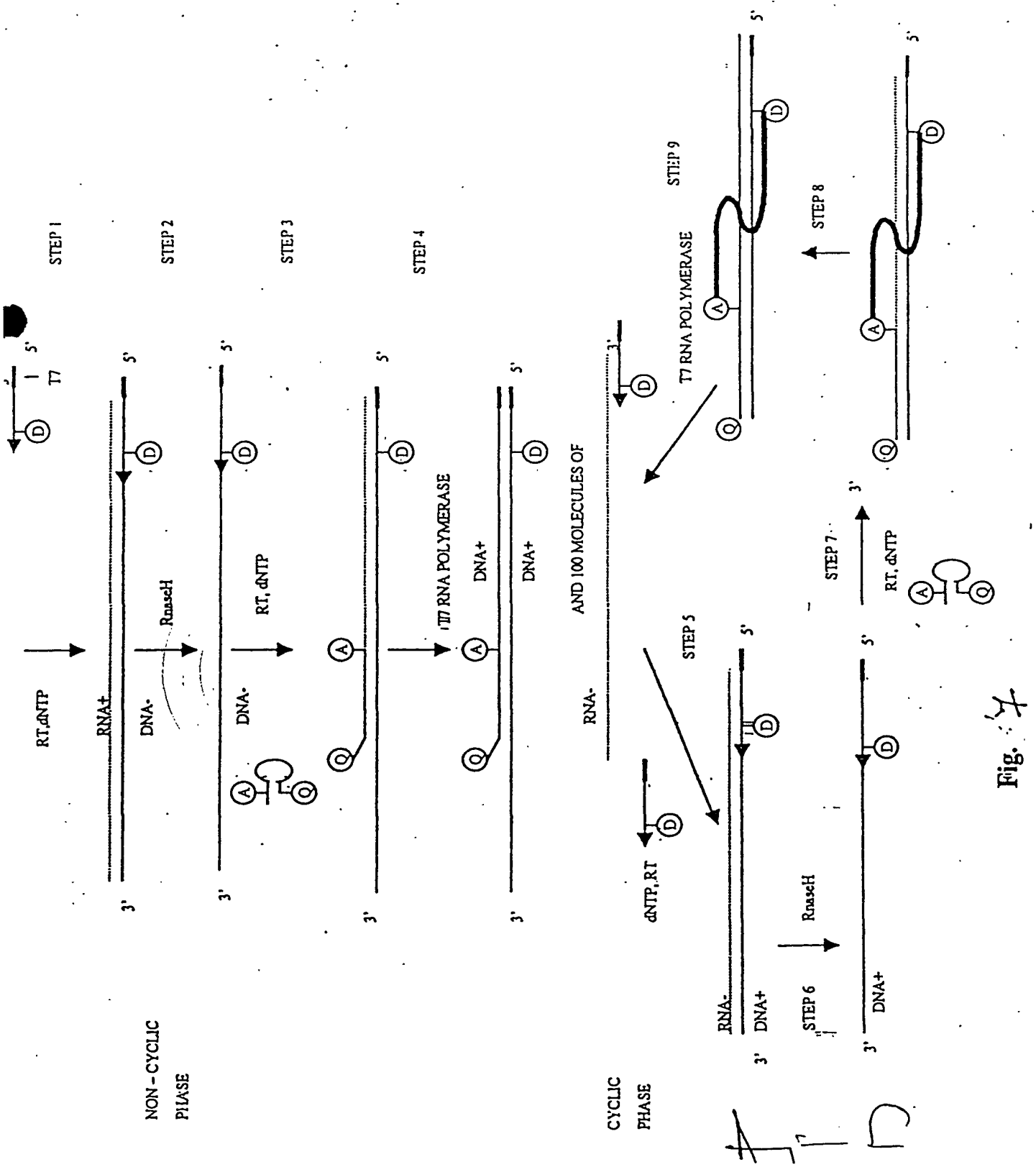

Fig. 4

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5'-GTT TTT GTG GTA GTA TGT GAT TTA GTC ATT CAA CCG TCG TAG TGG GCG CAA ACG CTC TAA CTT AAG TGT A-3'

Fig 8

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5'-TGC GGG GTA CTA CAG CGC CCT GAC CAT GGC CAT CTT CCA GGA CCT CGG-3'

Fig 9

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5'-ACG GAG CGG CTG AAG GTG CGG CAG GTG CAG GAC AAG TGG A-3'

Fig 10

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5'-ATG GCG CCT GCC TCG GAT GCG GGG TAC TAC AGC GCC-3'

Fig 11

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5'-ACT TAA GTT AGA GCG TTT GC-3'

|

FAM

Fig 12

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5'-GGG GTA CTA CAG CGC CCT GA-3'
|
FAM

Fig 13

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5' - GTC CTG GAA GAT GGC CAT GG-3'
|
JOE

Fig 14

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5' - GGG GTA CTA CAG CGC CCT-3'
|
FAM

Fig 15

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DABCYL-5'-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG-3'

|

JOE

Fig 16

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5'- DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG-3'
|
FAM

Fig 17

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5' -GCT CAT GGC GCC TGC CTC G-3'
|
DABCYL

Fig 18

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5'-TGCACACGGA GCGGCTGAAG GTGCGGCAGG TGCAGGACAA
GTGGAAGGTG ACGGGCATGG GCAACGAGAT CTGTGGCCAC
TTCAAGGTGC CGCCGGCGCA CATCACCGAT GGCCTGAGCA
ACACCGACTT CGTGATGTAC GTCGCCTCCG TGCCGAGCGA
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CGGCGAACAT TGCGTCGCGG TACGACCAGC TGGTGACGCG
TGTCGTCACG CACGAGATGG CGCACGCGCT CGGCTTCAGC
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CAGCAGCACG GCGGTGGCGA AGGCGCGCGA GCAGTACGGC
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GTGCGGGCTC CGCCGGGTCG CACATCAAGA TGCGCAACGC
GCAGGACGAG CTCATGGCAC CTGCCTCGGA TGCGGGGTAC
TACAGCGCCC TGACCATGGC CATCTTCCAG GACCTCGGCT
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Fig 19

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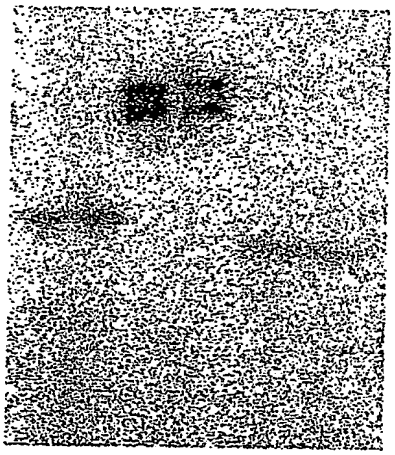

Fig. 20

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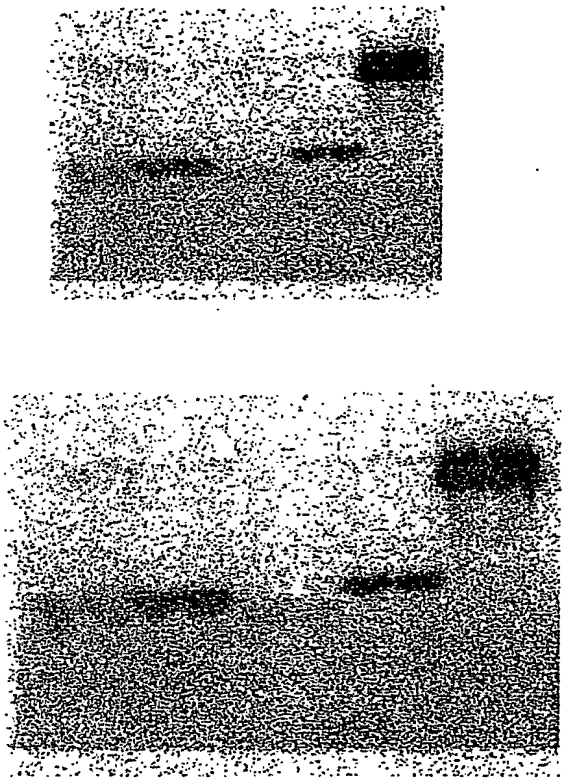

Fig.21

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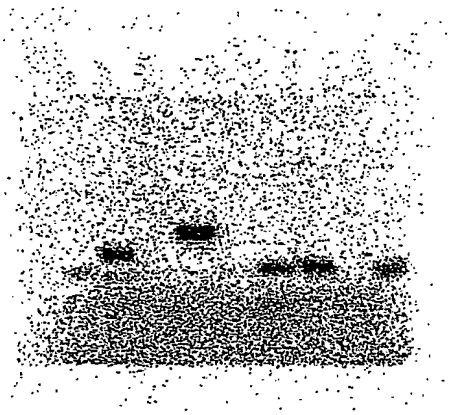

Fig.21A

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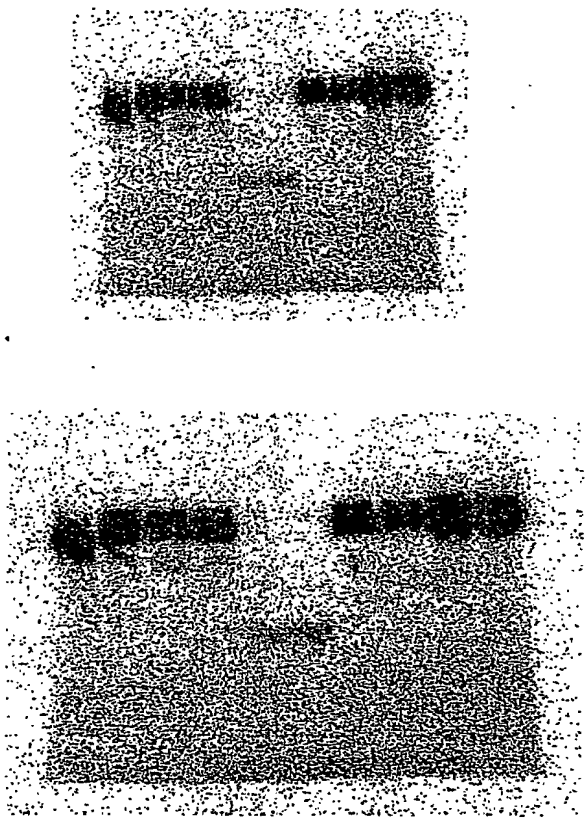

Fig.22

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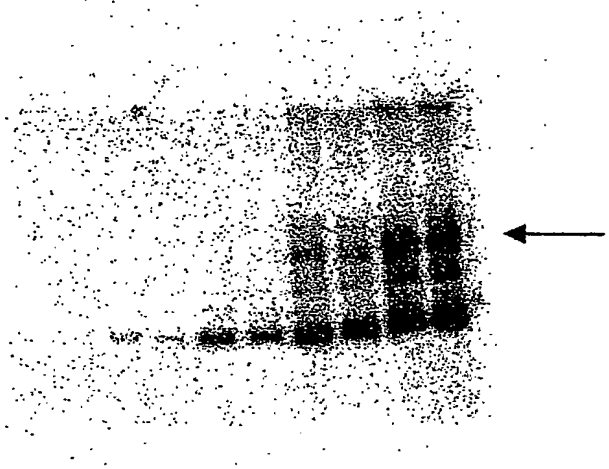

Fig.23

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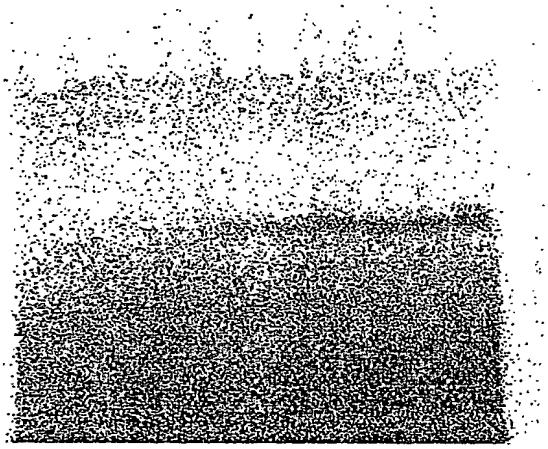

Fig.24

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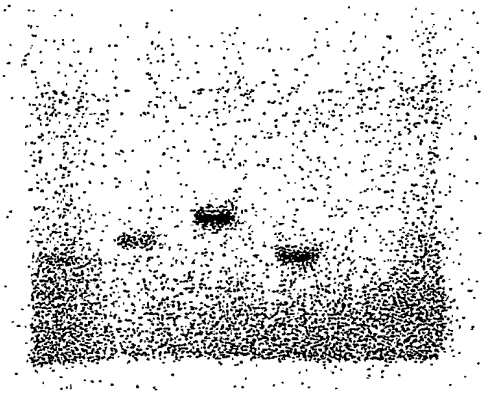

Fig.25

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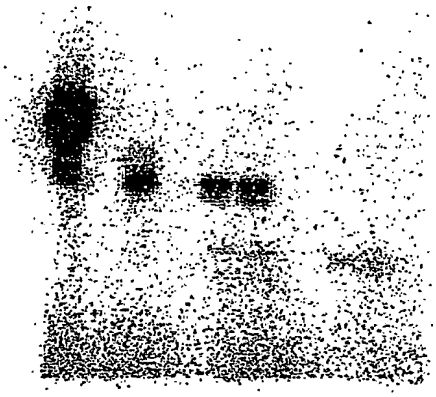

Fig.26

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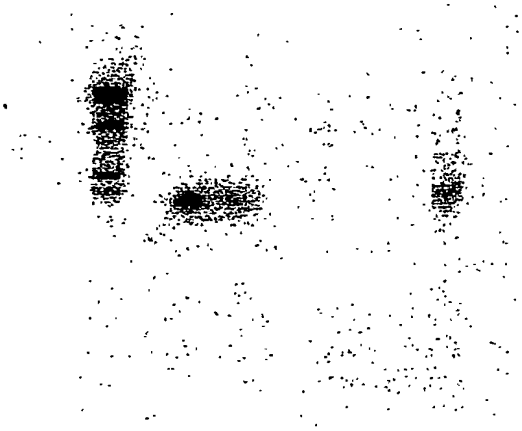

Fig.27

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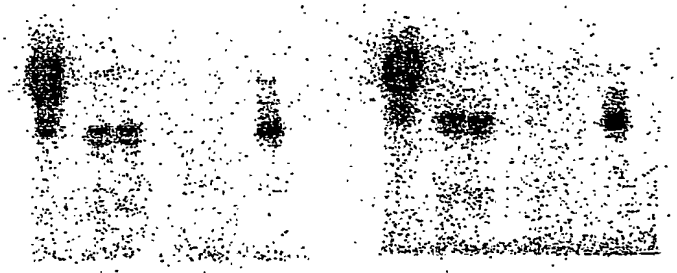

Fig.28

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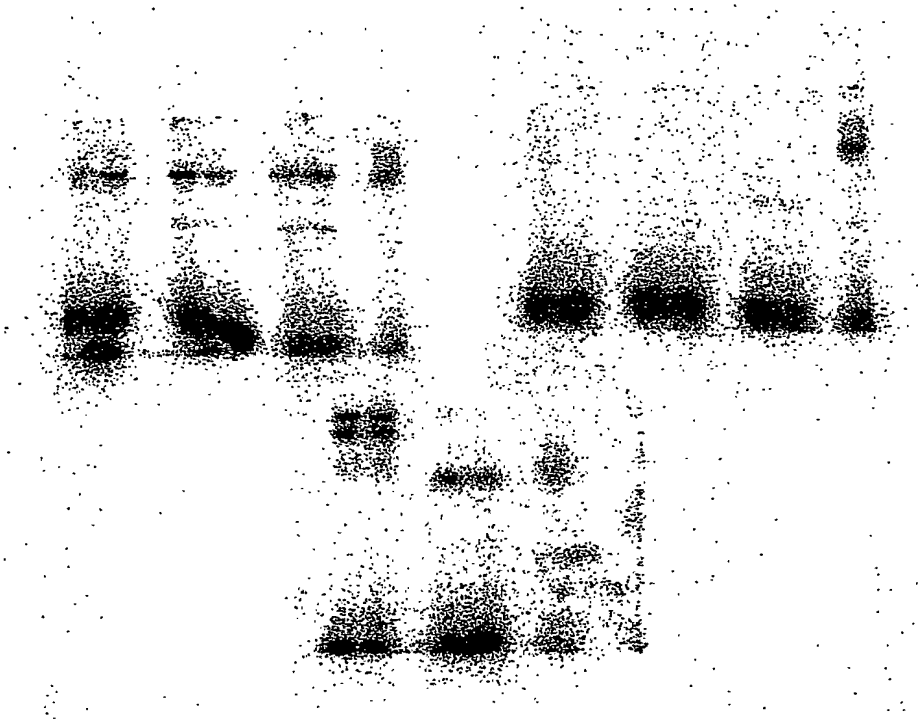

Fig.29

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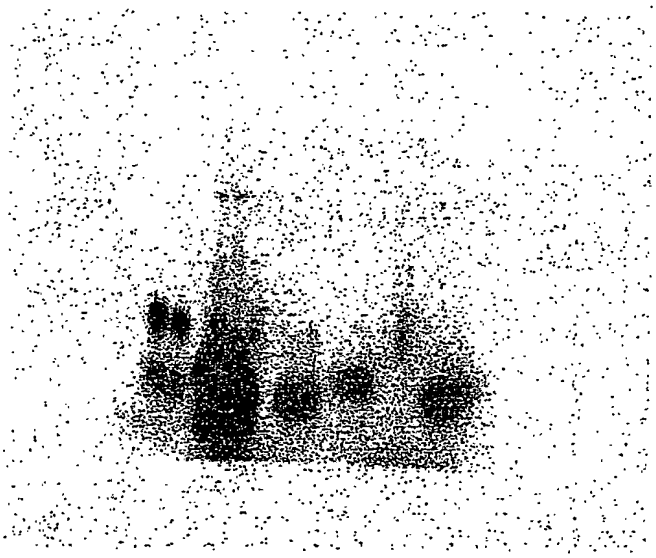

Fig.30

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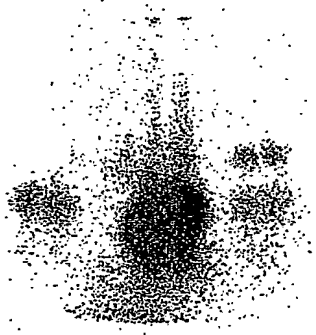

Fig.31

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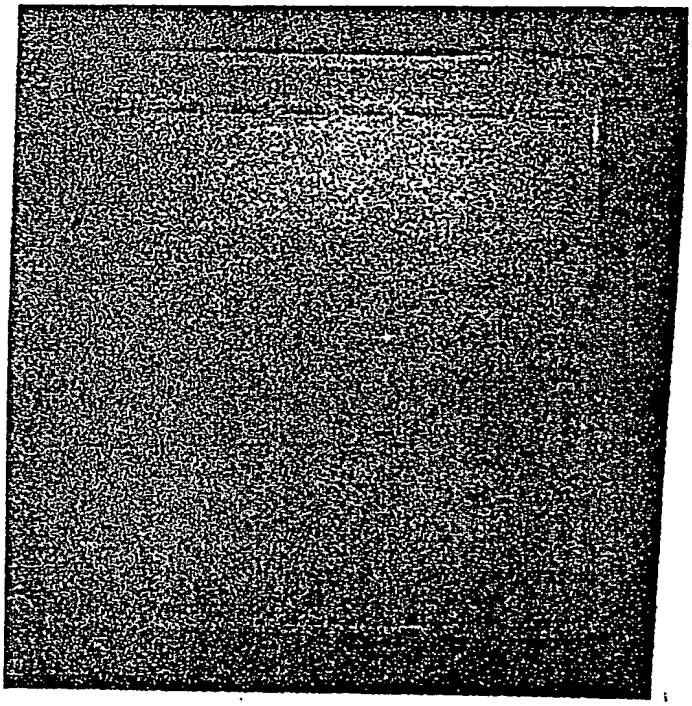

Fig. 32

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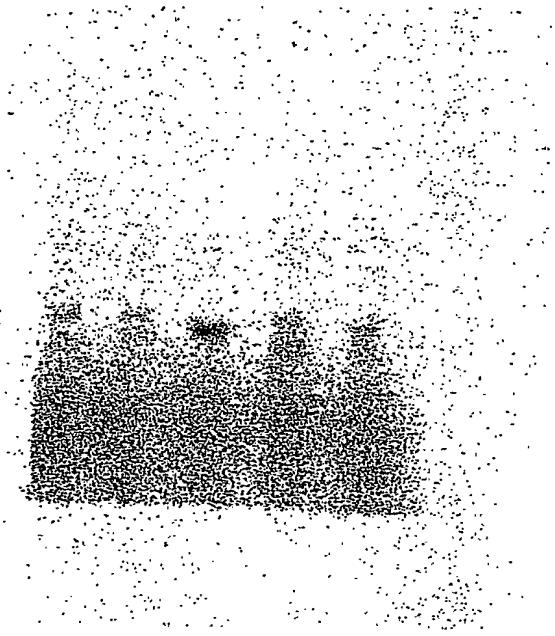

Fig.33

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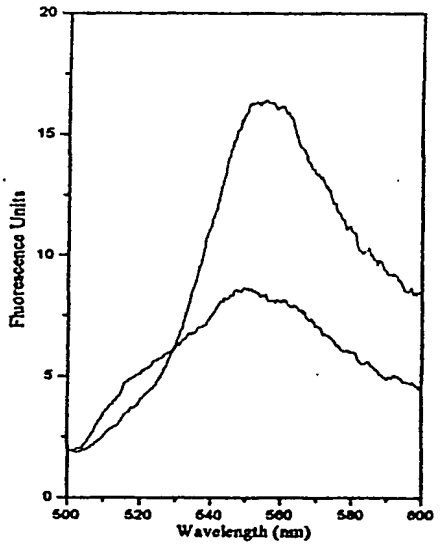

Fig. : 34

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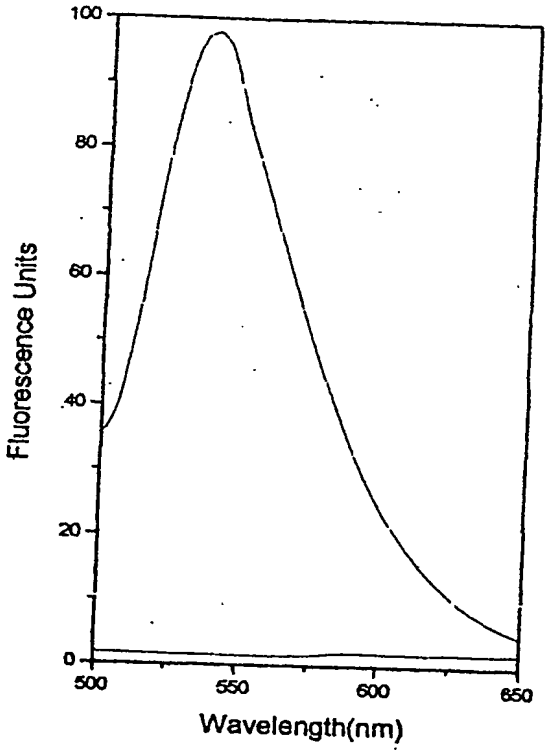

Fig. 35

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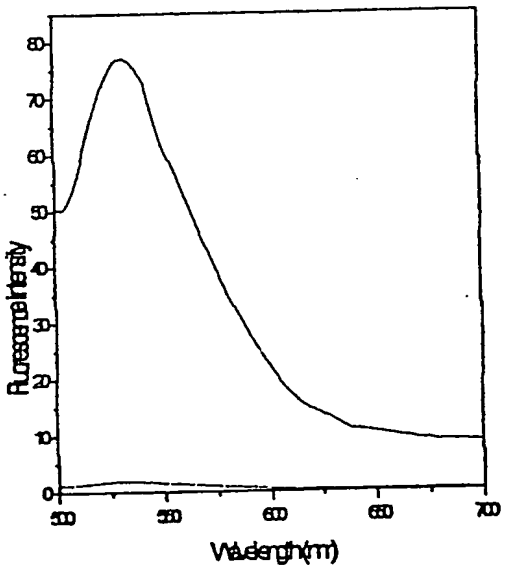

Fig- 36

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